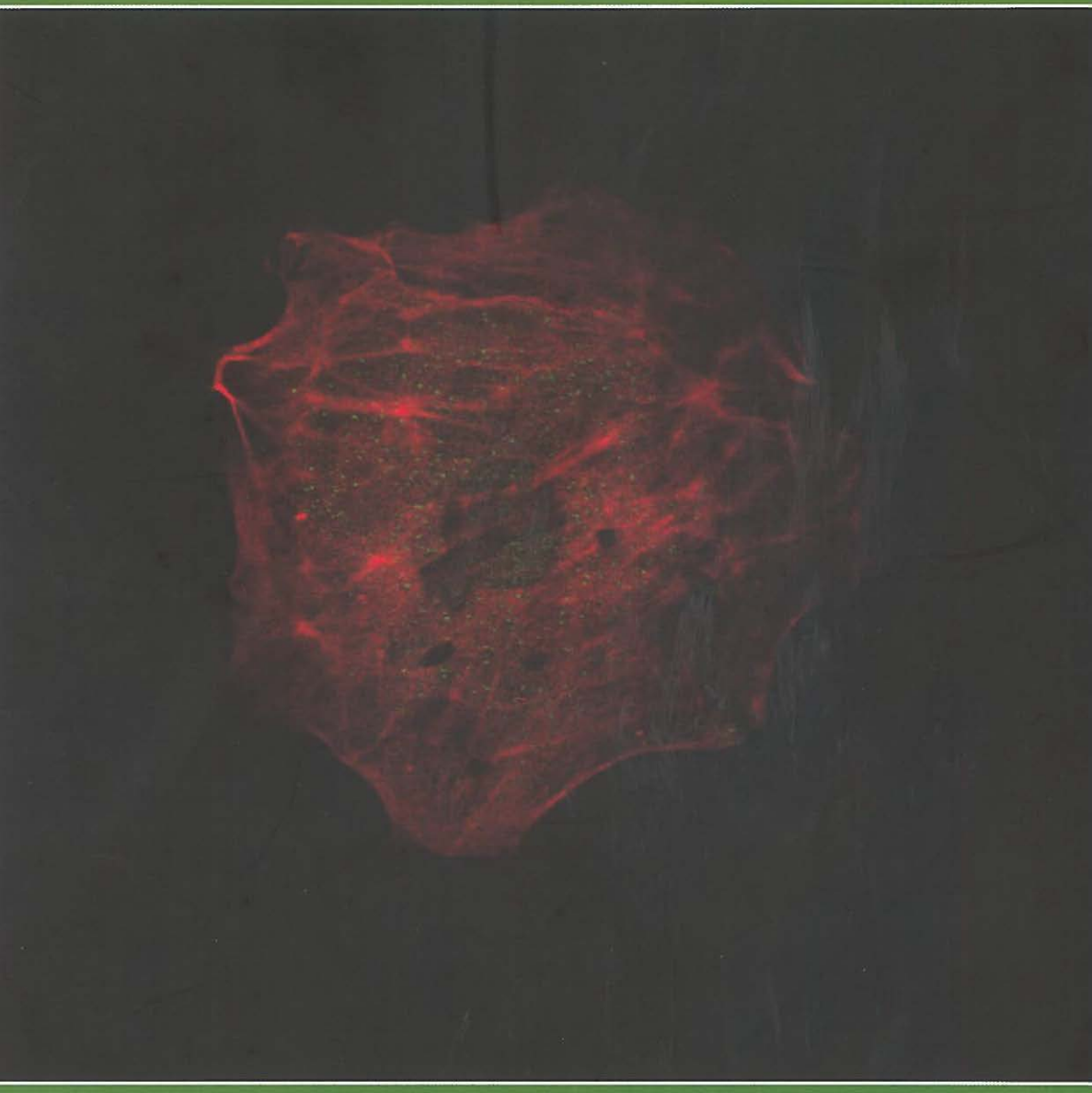
Resistance of activated stellate cells to cell death in liver fibrosis: mechanisms and targets for intervention



Sandra Dunning

Resistance of activated stellate cells to cell death in liver fibrosis: mechanisms and targets for intervention

Sandra Dunning

Stellingen

Behorende bij het proefschrift

Resistance of activated stellate cells to cell death in liver fibrosis: mechanisms and targets for intervention

door Sandra Dunning

De overeenkomsten in ABC-transporter expressie tussen stellaatcellen en progenitor cellen versterken het idee dat stellaatcellen ook progenitor cellen zijn.
Kordes et al. Biochem Biophys Res Commun 2007;352:410-417 en dit proefschrift

Door een meting van de metabole activiteit kan men niets zeggen over de proliferatie of vitaliteit van cellen.
Dit proefschrift

Het geven van antioxidanten bij leverfibrose zal niet alleen gunstige effecten hebben.
Dit proefschrift

Galzuur geïnduceerde proliferatie van stellaatcellen verloopt via de angiotensine II type 1 receptor en de epidermal growth factor-receptor.
Eigen observatie

Het geneesmiddel ribavirine remt de proliferatie van stellaatcellen en dient daarom als nieuwe therapeutische optie voor niet-virale leverfibrose nader onderzocht te worden.
Dit proefschrift

Wat u ziet is geen realiteit maar de projectie van uw eigen gedachte.
Anoniem

Toegeven dat u iets niet weet is geen schande, het is eerlijk.
Anoniem

Je weet nooit waar je acties toe leiden. Maar als je niets doet, leiden ze nergens toe.
Mahatma Gandhi (1869-1948)

Doe waar je in gelooft en geloof in wat je doet. De rest is verspilling van tijd en energie.
Nisargadatta

Cover picture: Confocal laser scanning microphotograph of rat stellate cell treated with gliotoxin. Green corresponds to α-smooth muscle actin, red corresponds to active caspase-3.

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Chapter 1

General introduction

Scope of the thesis

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General introduction

The Liver

The "building block" of the normal liver is the liver lobule. Each lobule is supplied by blood from the portal vein, mixed with blood from the hepatic artery. The blood flows to the central vein (hepatic venule) via the liver sinusoids that are lined by a discontinuous, fenestrated layer of endothelial cells. Exchange of nutrients and metabolites takes place at the level of the sinusoids. Interspersed between the endothelial cells are the Kupffer cells, the liver-specific macrophages, which play an important role in the innate immune defense of the liver. Hepatocytes are the functional liver cells, and produce bile which is transported via the bile canaliculi to the bile duct and is eventually collected in the gallbladder. The space between the endothelial cell layer and the hepatocytes is called the space of Disse, where the hepatic stellate cells are located (1) (figure 1).

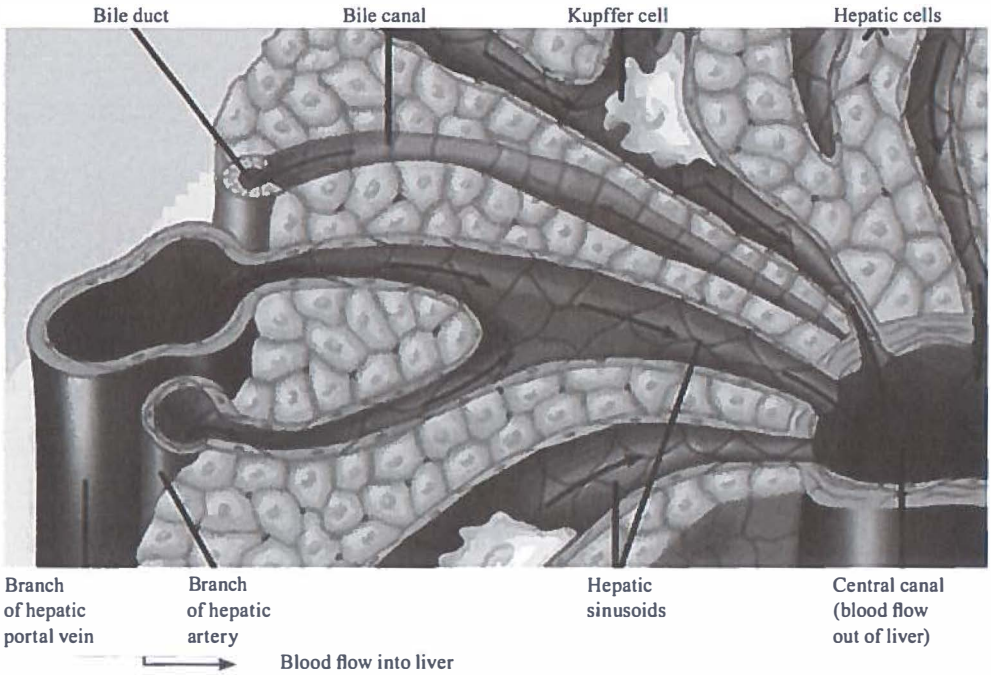

Figure 1. Detail of liver lobule (reprinted with permission from http://www.daviddarling.info).

Liver fibrosis

Fibrosis is the result of a sustained wound healing response in the liver (2). Acute injury will also activate the wound healing response, but only chronic or sustained injury will lead to fibrogenesis and liver fibrosis (3-5). Liver cirrhosis, the most advanced stage of liver fibrosis, is often accompanied by liver cancer and is therefore the most lethal among hepatobiliary and digestive diseases (4). Liver cirrhosis affects blood flow (1), oxygenation and metabolic and detoxification capacity of the liver (3). Currently, there is no effective therapy to treat liver fibrosis or liver cirrhosis. Liver transplantation is the only effective therapy, but has its limitations and disadvantages, like organ shortage, peri- and postoperative mortality and recurrence of viral infections in the donor liver (6).

A hallmark of liver fibrogenesis is the excessive formation and deposition of extracellular matrix (or scar tissue) in the liver (6) (figure 2). This excessive deposition of scar tissue leads to capillarization of the sinusoids and impaired uptake of nutrients and oxygen from the sinusoids. Together with the sustained injury and accompanying inflammation, this will lead to death of hepatocytes, the functional parenchymal liver cells and hence elevated serum levels of aspartate aminotransferase (AST) and alanine aminotransfertase (ALT). In advanced stages of fibrosis, characterized by portal-portal and portal-central bridging fibrosis, the number of hepatocytes, i.e. the effective liver mass, can be significantly decreased, leading to impaired liver function or even (acute) liver failure.

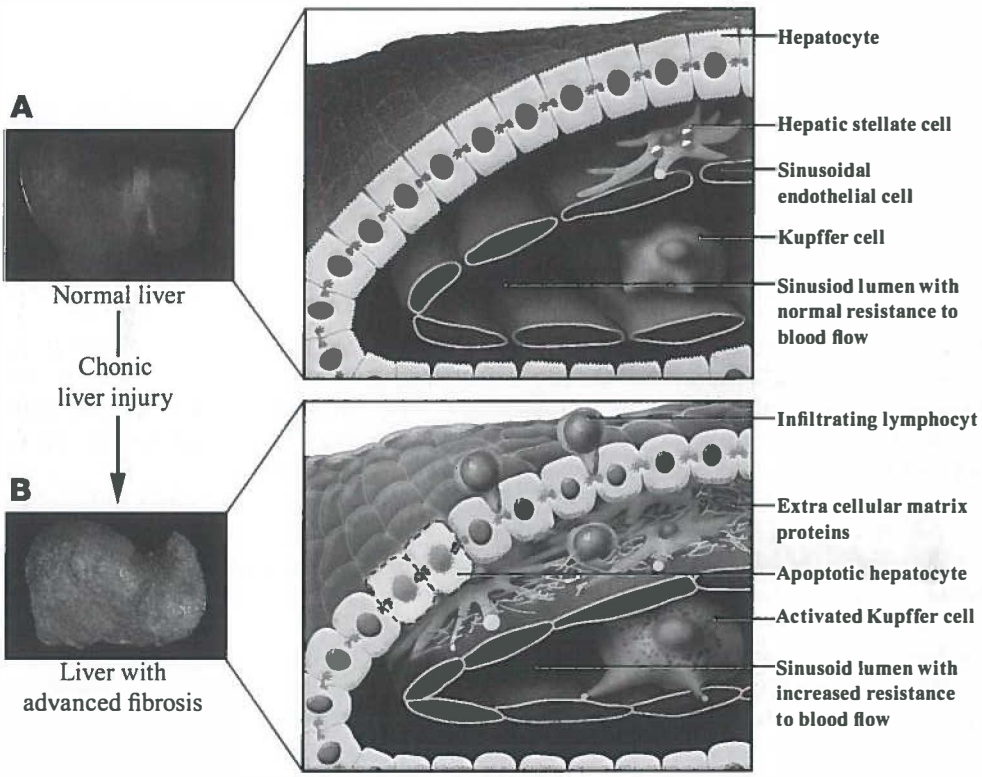

Figure 2. Changes in the hepatic architecture (A) associated with advanced hepatic fibrosis (B). During chronic liver injury, inflammatory cells infiltrate the hepatic parenchyma. Some hepatocytes undergo necrosis and/or apoptosis. Kupffer cells become activated and release fibrogenic mediators. Hepatic stellate cells (HSCs) proliferate and undergo a dramatic phenotypical activation, secreting large amounts of extracellular matrix proteins. Sinusoidal endothelial cells lose their fenestrations, and the contraction of HSCs causes increased resistance to blood flow in the hepatic sinusoid (5, reprinted with permission of the publisher).

Liver fibrosis and cirrhosis are common consequences of many chronic liver diseases of various etiology, including non-alcoholic and alcoholic fatty liver diseases, viral hepatitis and auto-immune related chronic liver diseases, such as auto-immune hepatitis, primary biliary cirrhosis and primary sclerosing cholangitis.

Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH)
Both NAFLD and NASH are characterised by increased macrovesicular fat deposition in the

hepatocytes, but only NASH is accompanied by overt signs of inflammation and elevated aminotransferases (AST and ALT) (7). NAFLD and NASH are associated with obesity and insulin resistance (8, 9). In NASH the lesions are mainly observed in the pericentral area (zone 3) of the liver lobule (10). In this area, the highest content of detoxifying oxygenating enzymes, like cytochrome P450-family members are found, making this area more likely to sustain oxidative stress, lipid peroxidation and injury (3, 11).

Alcoholic liver disease

Chronic alcohol abuse can lead to alcoholic steatohepatitis and liver fibrosis. The histopathological picture resembles (but is not identical to) that of NASH. Alcoholic steatohepatitis is also characterized by macrovesicular fat deposition (12). The detoxification of alcohol is accomplished by alcohol dehydrogenase and an inducible isoform of the cytochrome P450-family, CYP2E1 (13, 14). The metabolism of alcohol is characterized by the generation of reactive oxygen species and oxidative stress, again leading to lipid peroxidation and sustained inflammation and injury (13, 14).

There is no effective therapy available for alcoholic and non-alcoholic liver diseases. Current therapy includes immunosuppressive (15) or anti-inflammatory therapy, e.g corticosteroids (12) and in case of NAFLD/NASH also insulin-sensitizing agents like metformin (16) and anti-TNF (16) are in the experimental phase.

Viral hepatitis

Both the hepatitis B and hepatitis C virus can cause chronic hepatitis, evolving into fibrosis and eventually cirrhosis. The latency period can be very long, more than 10 or even 15 years (4, 17). In some groups of patients infected with hepatitis C virus (HCV) fibrosis will progress more rapidly, e.g. in re-infected patients after liver transplantation, HCV-HIV co-infection or chronic hepatitis C in conjunction with excessive alcohol consumption (10, 18).

The current treatment of hepatitis C patients is aimed at eradicating the hepatitis C virus using a combination of interferon alpha-2b and ribavirin (4, 19-21). However, this therapy is only effective in 50% of patients and the success rate is dependent on the genotype of the virus (21). Novel therapies, including viral protease inhibitors are in experimental stages (22).

Autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis

Auto-immune hepatitis, primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) are thought to be auto-immune-like liver disorders. The chronic immune response against auto-antigens in the liver leads to sustained inflammation and fibrogenesis (11). In case of PBC, the auto-immune response is directed towards the E2 component of the mitochondrial pyruvate dehydrogenase complex (23). The pathogenesis and auto-antigens of auto-immune hepatitis and PSC are less well characterized (24, 25).

Current therapeutic options for auto-immune liver diseases include corticosteroid therapy (26), immunosuppressive drugs like azathioprine or cyclosporine (27), and ursodeoxycholic acid (11, 25, 27). However, the clinical course of these disorders, despite therapy, is usually progressive and eventually requires liver transplantation.

The Hepatic Stellate Cell

The principal cell type involved in fibrogenesis and the excessive deposition of extracellular matrix during fibrogenesis is the hepatic stellate cell (28). Stellate cells were first discovered by the German anatomist Karl Wilhelm von Kupffer in the 19th centrury. Since its discovery, many different names were given to the stellate cell: 'Ito cell', 'lipocyte', 'vitamin A storing cell',

sternzellen (star cells in German) (29, 30), 'perisinusoidal cell' and 'fat-storing cell'. In 1996 the name was standardized to 'hepatic stellate cell'.
In the healthy liver the stellate cells store retinoids (vitamin A related compounds), produce small but appropriate amounts of normal extracellular matrix that is deposited as the basal lamina, and produce appropriate amounts of growth factors and cytokines (31).

Stellate cells in liver fibrosis
One of the hallmarks of liver fibrosis is the activation of stellate cells and their transformation into a myofibroblast-like cell type (2, 3, 5, 11, 30). Early events in the process of activation, the initiation phase, are rapid changes in gene expression, e.g. a rapid increase in the expression of α-smooth muscle actin (an important component of stress fibers), transforming growth factor-β and genes coding for extracellular matrix components like collagen type 1. These changes are believed to sensitize the stellate cell to the action of cytokines, reactive oxygen species (ROS), growth factors and other stimuli (2). Sources of ROS within the fibrotic liver are hepatocytes, Kupffer cells (the resident macrophages of the liver), and infiltrating inflammatory cells (32). Endothelial cells can also affect stellate cell activation, since injured sinusoidal endothelial cells produce a splice variant of cellular fibronectin, that in turn can activate HSCs (33).
In the next phase of activation, the perpetuation phase, the stellate cell will change further under the influence of cytokines, growth factors and remodelled extracellular matrix (ECM). During this phase, the normal low density ECM is replaced by a high density fibrillar collagen-rich ECM, in particular collagens type I and III. This fibril-rich collagen will accelerate stellate cell activation but will also affect the hepatocytes and sinusoidal endothelial cells (2, 5).
Furthermore, stellate cells in this phase also display increased proliferation, contractility, chemotaxis, retinoid loss, and release of cytokines and chemo-attractants (figure 3) (2). Proliferation of stellate cells is accomplished by several growth factors, the most important and potent being platelet derived growth factor (PDGF) (34). The proliferation-stimulating effect of PDGF is also observed in vitro (35). PDGF also has effects on chemotaxis of activated stellate cells (35, 36).
Resolution of liver fibrosis has been demonstrated in human (37, 38) and animal studies (39, 40). Resolution of liver fibrosis could be accomplished by diminishing the number of activated HSCs. This implies either a reversal of the activated phenotype into the quiescent phenotype, or the removal of activated stellate cells by inducing its death. It is questionable whether the activated HSC can revert into its quiescent phenotype in vivo. In vitro, reversal has been observed by plating the cells on a basement membrane matrix (41, 42), or replenishing its retinoid stores (43-45). Many papers stress the importance of stellate cell apoptosis in vivo in the (spontaneous) recovery of fibrosis (46, 47). It is important to note that activation of stellate cells has been observed in many human chronic liver diseases, including viral hepatitis, NASH and hemochromatosis (48-52).

Survival of hepatic stellate cells in liver fibrosis
Stellate cells survive in the hostile environment of the fibrotic liver. A similar survival in a hostile environment has been described for hepatic progenitor cells (53, 54). These are precursor cells that expand into cells of a specific lineage to restore the number of these cells. In conditions of massive liver cell death (e.g. acute liver failure), the hepatic progenitor cell compartment is activated and expands, restoring the functional liver mass (55). An important role in the survival and expansion of this population of hepatic progenitor cells is played by Mdr- and Mrp-type ATP-binding cassette (ABC) transporters (53). These are transmembrane transporters that export potentially toxic metabolites out of the cell (56). Like hepatic progenitor cells, the activated

stellate cells are also able to proliferate under toxic conditions (55). Since the expression of ABC-transporters allows the progenitor cells to survive in the fibrotic liver, it is of interest to know the expression, regulation and function of these ABC-transporters in (activated) stellate cells. Currently, there are no data on the role of ABC-transporters in hepatic stellate cell biology.

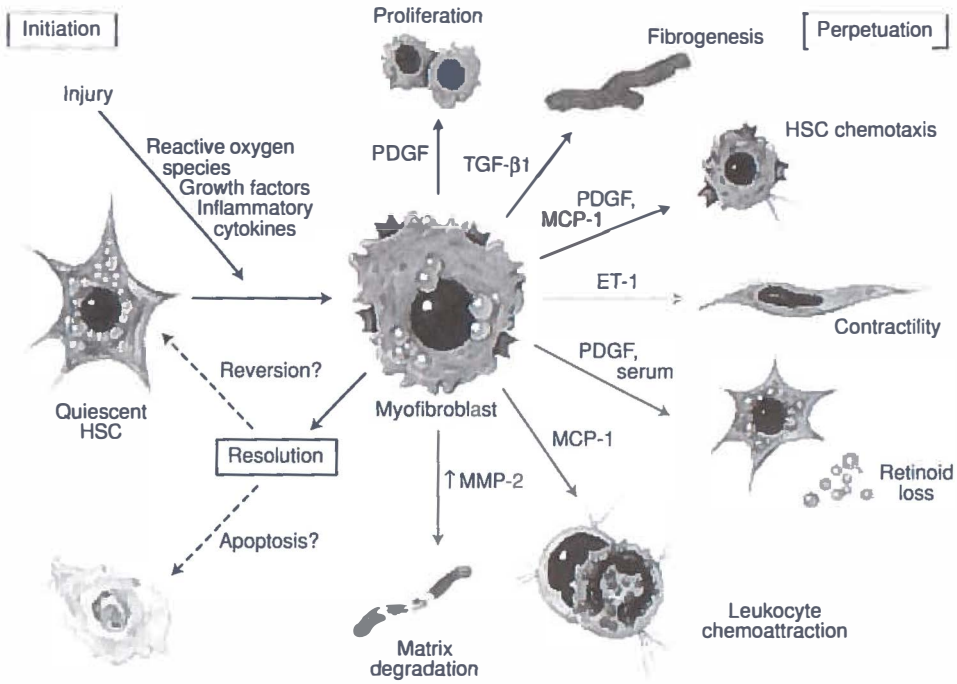

Figure 3. Phenotypic features of hepatic stellate cell activation during liver injury and resolution. Following liver injury, hepatic stellate cells become activated and transform from a quiescent vitamin A-rich cell into proliferative, fibrogenic, and contractile myofibroblasts. Important phenotypic alterations include proliferation, contractility, fibrogenesis, matrix degradation, chemotaxis, retinoid loss and chemoattraction. Key players mediating these alterations are indicated (2, reproduced from Friedman SL. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. J Biol Chem 2000;275(4):2247-2250).

Oxidative stress and stellate cell activation

Numerous studies have reported a correlation between the occurrence of oxidative stress in the liver and fibrogenesis and the activation of stellate cells (57, 58). Indeed, several experimental animal studies have shown that anti-oxidants can inhibit or reverse fibrogenesis (59-62). Furthermore, several studies have shown that oxidative stress can directly activate stellate cells and stimulate their proliferation (58, 63-68). However, opposite effects have also been observed: several studies have reported inhibitory effects of oxidative stress on stellate cell proliferation and matrix production and even death-inducing effects (69-71). The reasons for these discrepancies are not clear, but may be related to differences in ROS-generating systems, dosage and location of ROS-production. Nevertheless, the simultaneous presence of oxidative stress and the activation and proliferation of stellate cells in liver fibrosis are undisputable. Therefore, (activated) stellate cells must be unusually resistant to any detrimental effects of oxidative stress. The elucidation of the effects of oxidative stress on stellate cells is an important topic of this thesis.

Reactive oxygen species

Reactive oxygen species (ROS) or reactive oxygen intermediates (ROI) are small, highly reactive, oxygen-containing molecules that are naturally generated in small amounts during normal cellular metabolism (72). ROS are unstable due to the presence of unpaired electrons and therefore they are highly reactive and able to react with complex cellular macromolecules like fats (fatty acids), proteins, or DNA (72, 73). Examples of ROS are superoxide anions ($O2^{\cdot-}$), hydrogen peroxide (H2O2), and the hydroxyl radical ($^{\cdot}OH$).

The major source of ROS in the cell is the mitochondrial respiratory chain. Another major source of ROS, especially in the liver, is a group of enzymes called the cytochrome P450 mixed function oxidases (74). These enzymes are involved in the detoxification of endogenous and exogenous compounds. Increased generation of ROS occurs when cells are exposed to endogenous and exogenous compounds that need to be detoxified. The metabolism of alcohol by the cytochrome-P450 family member CYP2E1 is known to generate large amounts of ROS (13, 75). In addition, the metabolism of organic solvents, e.g. CCl4 or drugs, e.g acetaminophen, also generates large amounts of ROS. Furthermore, cells are exposed to environmental ROS, e.g. in smog (nitric oxide), and tobacco smoke, or environmental factors that can induce ROS, e.g. UV radiation (72). Finally, virtually all chronic liver diseases are accompanied by sustained inflammation and therefore the presence of (infiltrating) inflammatory cells like macrophages, Kupffer cells and neutrophils. These cells are important sources of ROS in chronic liver diseases (73, 74).

Defence mechanisms against reactive oxygen species

Because ROS are generated both under normal conditions and during (inflammatory) diseases, cells have to protect themselves against ROS. To this end, cells have developed several protective mechanisms, both enzymatic (ROS-detoxificating enzymes like catalase and superoxide dismutases) and non-enzymatic (anti-oxidants like glutathione and vitamin E) (72, 73).

Superoxide dismutases

Superoxide dismutases (SODs) are metal-containing enzymes that detoxify superoxide anions. There are several SODs: CuZn-SOD, MnSOD and extracellular EC-SOD. The CuZn-SOD (SOD1) is localized in the cytosol, the Mn-SOD (SOD2) is located in the mitochondria and the EC-SOD (SOD3) is found in the extracellular space (76, 77). Superoxide dismutases convert (dismutate) superoxide anions into hydrogen peroxide.

$$2\,O_2^{\cdot-} + 2\,H^+ \xrightarrow{\textbf{SOD}} H_2O_2 + O_2$$

Hydrogen peroxide detoxifying enzymes

Catalase is a hydrogen peroxide-detoxifying enzyme that is very abundant in the liver (hepatocytes). Within cells, catalase is located exclusively in the peroxisomes. Catalase converts hydrogen peroxide into water and oxygen (72).

$$2\,H_2O_2 \xrightarrow{\textbf{Catalase}} H_2O + O_2$$

Another enzyme that detoxifies hydrogen peroxide is the selenium containing enzyme glutathione peroxidase (GPx). There are four known mammalian GPxs. GPx1 can also metabolize organic peroxides, including long-chain fatty acid peroxides. GPx requires reduced glutathione (GSH) to perform its enzymatic reaction, yielding water and oxidized glutathione (78). Glutathione is

present both in the cytosol and in the mitochondria. The mitochondria are not able to produce glutathione and therefore need to import it from the cytosol.

$$2\ GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2\ H_2O$$

To control the hydrogen peroxide level within the cell, the cell has to balance the activity of superoxide dismutases relative to catalase and glutathione peroxidases (77).

Anti-oxidants

Reduced glutathione is an important intracellular anti-oxidant present in the cell. Besides being a component of the GPx-mediated reactions, it is also important in the conjugation of several ROS or other radicals, facilitating their export from the cell (79, 80).

Other important nonenzymatic anti-oxidants are vitamin E (a-tocopherol) and vitamin C (ascorbate). Vitamin E is found in the lipid phase of membranes and, like other chemically related molecules, acts as a terminator of lipid peroxidation. During the reaction between vitamin E and a lipid radical, the vitamin E radical is formed, from which vitamin E can be regenerated in a reaction involving GSH and ascorbate (72).

Cell death and survival of hepatic stellate cells

Oxidative stress is defined as an imbalance between ROS exposure and ROS-detoxifying capacity (13, 72, 73). It occurs when exposure to ROS is excessive, or when ROS-detoxifying capacity is compromised. Oxidative stress, the inappropriate exposure to ROS, can induce cell death. Two types of ROS-induced cell death can be distinguished: necrotic (passive) cell death and apoptotic (programmed) cell death (74, 81).

Necrosis does not require energy; hence it is ATP-independent and is considered to be an unregulated form of cell death. Necrosis is characterized by cell swelling and membrane disruption. In contrast, apoptotic cell death is the result of a highly regulated death program and requires ATP. Characteristics of apoptosis include nuclear condensation and the formation of apoptotic bodies. There is no cell swelling and no disruption of the plasma membrane. An apoptotic death signal leads to activation of caspases, cysteine-aspartate specific proteases, that cleave essential cellular proteins (81). Two types of apoptotic caspases are distinguished: initiator (apical) caspases and effector (executioner) caspases. Initiator caspases (e.g. CASP2, CASP8, CASP9 and CASP10) cleave inactive pro-forms of effector caspases, thereby activating them. Effector caspases (e.g. CASP3, CASP6, CASP7) in turn cleave other protein substrates within the cell resulting in apoptosis (82).

Apoptosis of stellate cells can be induced by gliotoxin, a fungal metabolite. Gliotoxin-induced apoptosis is dependent on caspase 3 activation and addition of the caspase inhibitor z-VAD-FMK reduced oligonucleosomal DNA fragmentation, characteristic of apoptotic cell death (83, 84). An in vivo study, using the CCl4 –intoxication model of liver fibrosis, showed that a single injection of gliotoxin accelerated resolution of fibrosis (83). This was demonstrated by a reduction in the number of activated HSC and in the reduction of the thickness of the fibrotic septae. The reduction of the number of stellate cells was shown to be caused by apoptosis (83). In contrast, the endogenous cannabinoid anandamide induces necrotic death of stellate cells (85).

An important role in the regulation of cell death and survival is played by a family of kinases known as mitogen-activated protein (MAP) kinases or stress-activated protein kinases (SAPKs). These are serine/threonine-specific protein kinases that respond to extracellular stimuli, like oxidative stress, and regulate numerous cellular activities, such as gene expression, mitosis,

differentiation, and cell survival and apoptosis. They act via a protein phosporylation kinase cascade. MAP kinases may have overlapping substrate specificities. Mitogen-activated protein kinases (MAPK) have been shown to be involved in HSC proliferation: Ras activation followed by the sequential activation of Raf, mitogen-induced extracellular kinase (MEK), and extracellular signal regulated kinase (ERK) is one signaling cascade that is activated by PDGF in HSC, both in vitro and in vivo (86-89).
Whereas the MAP kinase ERK usually has an anti-apoptotic and/or proliferative effect, the MAP kinases c-Jun N-terminal kinases (JNKs) in general have pro-apoptotic and/or anti-proliferative effects. However, it should be noted that an evaluation of the effects of the bile acid deoxycholic acid on hepatocytes isolated from jnk1-/- or jnk2-/- mice, indicated the presence of JNK isoform-specific functions in hepatocyte cell death. Specifically, JNK2 signalling was cytoprotective whereas JNK1 signalling was cytotoxic (90). This dual role of JNKs in cell death and survival has not been investigated yet in hepatic stellate cells.
Finally, inhibition of p38, another MAPK member, using the pharmacological inhibitor SB-203580 in either quiescent or activated HSC, increased cell proliferation, indicating that activation of p38 inhibits HSC proliferation (91).This inhibitory role for p38 in cell proliferation has been shown in other cell types and may be due to inhibition of cyclin D1 expression (92).

Scope of the thesis

Liver fibrosis is caused by sustained injury to the liver, which results in death of hepatocytes and activation and proliferation of hepatic stellate cells. Within the liver many factors are involved in the activation of hepatic stellate cells and death of hepatocytes. Activation of stellate cells is detrimental since these cells produce excessive amounts of extracellular matrix, leading to e.g. portal hypertension. Fibrosis may be reversible when the activated stellate cells are forced into apoptosis or necrosis or if they reverse into the quiescent phenotype again. Understanding how the stellate cell can cope with potentially toxic factors like oxidative stress within the fibrotic liver is important to identify novel targets for intervention that interfere with the activation process or the survival of activated hepatic stellate cells.
In this thesis, several mechanisms underlying the resistance of hepatic stellate cells against cell death are uncovered.
Chapter 2 highlights the role of Mrp- and Mdr-type transporters in activated stellate cells, in particular Mrp1. The expression and regulation of these transporters, both in vivo and in vitro are investigated and the importance of Mrp-type transporters in the survival of hepatic stellate cells is demonstrated. Mrp1 is an interesting target for intervention aimed at preventing or reversing liver fibrosis and cirrhosis.
Chapter 3 is a detailed analysis of the effects of oxidative stress on the viability of activated hepatic stellate cells. We demonstrate that reactive oxygen species do not directly induce stellate cell proliferation, but rather block stellate cell proliferation and induce cell death. Another important finding of this study is that superoxide anion-induced stellate cell apoptosis is caspase–independent and can be reversed by glutathione supplementation.
In **Chapter 4** the defence mechanism(s) of stellate cells against oxidative stress are investigated in detail. The most important findings of this study are that the protection against reactive oxidative stress is due, to a large extent, to a high intracellular glutathione content and the presence of active hydrogen peroxide detoxifying enzymes such as glutathione peroxidase-1 (GPx1). Furthermore, we demonstrate that these crucial protective mechanisms (glutathione and GPx1) are both enhanced in activated stellate cells compared to quiescent stellate cells.

Chapter 5 investigates the possible antifibrotic action of ribavirine, a drug administered to HCV infected patients to clear viral load. Although ribavirine is used as an antiviral drug, we demonstrate that ribavirine directly inhibits proliferation of activated stellate cells. In addition, we demonstrate the antifibrotic effect of ribavirin in a non-viral model of liver fibrosis (bile duct ligation).

In conclusion, this thesis describes various mechanisms that contribute to the resistance of hepatic stellate cells to oxidative stress. This knowledge may contribute to the development of novel therapeutic strategies that aim to reduce the number of activated stellate cells and hence, limit (or reverse) liver fibrosis.

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Chapter 2

Multidrug resistance-associated proteins are crucial for the viability of activated rat hepatic stellate cells

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Abstract

Background
Hepatic stellate cells (HSCs) survive and proliferate in the chronically injured liver. ATP-Binding Cassette (ABC) transporters play a crucial role in cell viability by transporting toxic metabolites or xenobiotics out of the cell. ABC transporter expression in HSCs and its relevance to cell viability and/or activation has not been reported so far.

Aim
The aim is to investigate the expression, regulation and function of Mrp-type and Mdr-type ABC-transporters in activated rat HSCs.

Methods
Rat HSCs were exposed to cytokines or oxidative stress. ABC-transporter expression was determined by qPCR and immunohistochemistry. HSCs were exposed to the Mdr-inhibitors verapamil and PSC-833 and the Mrp-inhibitor MK571. Mdr and Mrp transporter function were evaluated using flow cytometry. Apoptosis was determined by activated caspase-3 and acridine orange staining and necrosis by Sytox Green nuclear staining. An in vivo model of CCl4-induced liver fibrosis was used.

Results
Relative to hepatocytes, activated HSCs expressed high levels of Mrp1 and comparable levels of Mrp3, Mrp4, Mdr1a, and Mdr1b but not the hepatocyte-specific transporters Bsep, Mrp2 and Mrp6. Mrp1 protein staining correlated with desmin staining in livers from CCl4-treated rats. Mrp1 expression increased upon activation of HSCs. Cytokines induced Mdr1b expression only. Mdr- and Mrp-type transporter expression was not regulated under oxidative stress. Activated HSCs became necrotic when exposed to the Mrp-inhibitors.

Conclusion
Activated HSCs contain relatively high levels of Mrp1. Mrp-type transporters are required for the viability of activated HSCs. Mrp-dependent export of endogenous metabolites is essential for the survival of activated HSCs in chronic liver diseases.

Introduction

During chronic liver injury, liver cells are exposed to bile acids, oxidative stress and cytokines (1, 2). Under these conditions, hepatic stellate cells (HSCs) and hepatic progenitor cells (HPCs) proliferate (3-5), while hepatocytes die. Hepatocyte death leads to impaired liver function whereas HSC activation and proliferation leads to the development of liver fibrosis and cirrhosis (6). In recent years much progress has been made in elucidating the mechanisms involved in activation and proliferation of HSCs and the mechanisms of hepatocyte death induced by bile acids, oxidative stress, and cytokines (7-9). Also, recent studies provide evidence that liver fibrosis and even cirrhosis can be reversed by inducing hepatic stellate cell apoptosis (10). However, virtually nothing is known about the protection mechanisms of HSCs against the toxic agents that are present in chronic liver injury.

ATP-binding cassette (ABC) transporters play a major role in the multidrug resistant and proliferative phenotype of many cancer cells. ABC transporters also play an extremely important role in liver physiology. ABC transporters in the liver are involved in hepatobiliary transport of metabolites of endogenous and exogenous compounds, phospholipids, cholesterol and bile acids in hepatocytes, bile duct epithelial cells and enterocytes (11). Two subclasses of these ABC transporters Mdr-type transporters (multidrug resistance protein, ABCB subclass) and Mrp-type transporters (multidrug resistance associated protein, ABCC subclass) protect the proliferating cell from xenobiotics, including chemotherapeutics, and endogenous cell stress, by transporting toxic metabolites out of the cell (12). Mdr-type transporters are capable of removing many hydrophobic substrates from the cell, whereas Mrp-type transporters, in particular Mrp1, transport organic anions like glutathione, glucuronate and sulfate conjugates (13). Recently, high expression of several ABC transporters in HPCs has been described in various liver pathologies. Most prominent is the expression of Mdr1b, Mrp1, Mrp3 and BCRP in HPCs (14, 15). It is assumed that high expression of these transporters enable HPCs to survive liver injury and to proliferate, because these ABC-transporters export endogenous and exogenous toxic compounds out of the cells. Like HPCs, HSCs survive and proliferate in the chronically injured liver. However, nothing is known about the role of ABC transporters in HSCs. Therefore, the aim of the present study was to investigate the expression, regulation and function of ABC transporters in activated HSCs. Our study demonstrates that ABC transporters are expressed in HSCs and play an important role in the protection against cell death.

Materials and methods

Animals

Specified pathogen-free male Wistar rats were purchased from Harlan (Zeist, The Netherlands). They were housed under standard laboratory conditions with free access to standard laboratory chow and water. Each experiment was performed following the guidelines of the local committee for care and use of laboratory animals.

Liver fibrosis was induced in male Wistar rats, 250-300 g body weight, by administering CCl_4 for two weeks as described previously (16). Liver specimens were snap frozen in liquid nitrogen-cooled isopentane and stored at -70°C until use.

Rat hepatic stellate cell isolation and culture
Hepatic stellate cells (HSCs) were isolated from male Wistar rats (500-600 g) by pronase and collagenase perfusion of the liver, followed by Nycodenz gradient (12% w/v) centrifugation as described previously (17).
Cells were then cultured in Iscove's Modified Dulbecco's Medium with Glutamax (Invitrogen, Breda, Netherlands) supplemented with 20% heat-inactivated fetal calf serum (Invitrogen), sodium-pyruvate (Invitrogen), non-essential amino acids (Invitrogen), 50 μg/mL gentamycine (Invitrogen) and Nystatin 250 U/mL (Sanofi-Synthelabo, Maassluis, Netherlands) in a humidified atmosphere containing 5% CO2 at 37°C. Primary HSC cultures were allowed to grow to confluence, subcultured by trypsinization and then cultured in Iscove's medium with supplements as described above, but without Nystatin. Prior to experiments, HSC were serum-starved for 24 hours.

Human hepatic stellate cell isolation and culture
Human HSCs were extracted from the margins of normal human livers resected for colonic metastatic disease as previously described (18). Human HSCs were used for experimentation after activation and before the fourth passage. The use of human liver tissue for scientific investigation was approved by the UK South and West Local Research Ethics Committee and was subject to patient consent.

Hepatocyte isolation and culture
Primary rat hepatocytes were isolated from male Wistar rats (200–250 g) using a two-step collagenase perfusion as described previously (19). Cell viability was consistently more than 90% as determined by trypan blue exclusion. Isolated hepatocytes were plated at a density of 125000 cells per cm^2 in William's medium E (Invitrogen) supplemented with 50 μg/mL gentamycin (Invitrogen). During the attachment period (4 hours), 50 nmol/l dexamethasone (UMCG Pharmacy) and 5% fetal calf serum (Invitrogen) were added to the medium. Cells were cultured in a humidified incubator containing 5% CO2 at 37°C.

Experimental design
Culture-activated HSC (passage 2-3) were used for all experiments, unless indicated otherwise. Primary rat hepatocytes were used 18 hours after seeding.
Hepatocytes and HSC were exposed to a cytokine mixture (CM) containing 20 ng/mL recombinant mouse tumor necrosis factor α (mTNF-α, R&D Systems, Abingdon, United Kingdom), 10 ng/mL recombinant human interleukin-1β (hIL-1β, R&D Systems) and 10ng/mL recombinant rat interferon-γ (rIFN-γ, R&D Systems) for 6 hours. Oxidative stress was induced by exposing the cells to the intracellular superoxide anion donor menadione (2-methyl-1,4-naphthoquinone, Sigma Aldrich) or hydrogen peroxide (Merck, Haarlem, The Netherlands) at the indicated concentrations for 6 hrs. The LTD4 receptor antagonist MK-571 (Kordia Life Sciences, Leiden, Netherlands) was used as a Mrp-inhibitor (20). Verapamil (Sigma Aldrich) and the cyclosporin A analogue PSC-833 (Sigma Aldrich) were used as Mdr-inhibitors (21, 22).

Adenoviral constructs
The adenoviral constructs used were described previously (23). The IκB super-repressor adenovirus Ad5IκBAA contains an IκB construct in which serines 35 and 36 have been replaced by alanines. This mutated IκB cannot be phosphorylated and binds NF-κB irreversibly, preventing translocation of NF-κB to the nucleus. Ad5LacZ, expressing the Escherichia coli β-galactosidase gene, was used as a control virus throughout the experiments. Cells were

infected with adenovirus at the indicated multiplicity of infection (MOI) 24 hours before the start of the experiments.

RNA isolation, reverse-transcription-PCR and Real Time PCR

RNA was isolated using Tri Reagent (Sigma Aldrich) reagent according to the manufacturer's instructions. RNA was quantified using a Ribogreen fluorescent assay (Invitrogen). Reverse transcription and real-time PCR were performed as described previously (24). 18S RNA was used as an internal control. Primers and probes used for real-time PCR are listed in Tables 1 and 2.

Table 1. Sequences of rat PCR primers and probes used for real-time detection PCR analysis

Gene		Primers
18S	sense	5'-CGG CTA CCA CAT CCA AGG A-3'
	antisense	5'-CCA ATT ACA GGG CCT CGA AA-3'
	probe	5'-CGC GCA A AT TAC CCA CTC CCG A-3'
Mrp1	sense	5'-AGG CTT CTT GGC AAA TCC AA-3'
	antisense	5'-CAA GCA GTA ATC CCG GAA CTC T-3'
	probe	5'-TGG CCC CAT TCA GGC CGT G-3'
Mrp2	sense	5'-GAC GAC GAT GAT GGG CTG AT-3'
	antisense	5'-CTT CTC ATG GCC AAG GAA GCT-3'
	probe	5'-CCC ACC ATG GAG GAA ATC CCT GAG G-3'
Mrp3	sense	5'-TCC CAC TTC TCG GAG ACA GTA ACT-3'
	antisense	5'-CTT AGC ATC ACT GAG GAC CTT GAA-3'
	probe	5'-CAG TGT CAT TCG GGC CTA CGG CC-3'
Mrp4	sense	5'-TCA GTG TTG GAC AGA GAC AGT TAG TG-3'
	antisense	5'-CTT CTC CCG GAT TTT CTG TTG TAT-3'
	probe	5'-TCA GTT CTC GGA TCC ACA TTT GCA GTT G-3'
Mrp5	sense	5'-CGG CTA ACC GCG TAT TTC AG-'3
	antisense	5'-AAC GCT TTG ACC CAG GCA TA-3'
	probe	5'-TAG CCG CCA CAG ACG ACC GTG TC-3'
Mrp6	sense	5'-CTC TCC CAT TGG CTT CTT TGA G-3'
	antisense	5'-GTC CAC ATC CAC TAT GTC CGT CT-3'
	probe	5'-TCG GGA ACC TGC TGA ACC GTT TTT C-3'
Mdr1a	sense	5'-GCA GGT TGG CTG GAC AGA TT-3'
	antisense	5'-GGA GCG CAA TTC CAT GGA TA-3'
	probe	5'-CCG CCA GAG TTC CCA GCA GCA TG-3'
Mdr1b	sense	5'-AAA CAT GGC ACG TAA CCA AAG TT-3'
	antisense	5'-AAA ATG TGG CCC TGT TTA ATG ATT-3'
	probe	5'-CAC TGT TAA AGG TAA TTT CAT CAA GAC GAG AAG CCT TC-3'
iNOS	sense	5'-GTG CTA ATG CGG AAG GTC ATG-3'
	antisense	5'-CGA CTT TCC TGT CTC AGT AGC AAA-3'
	probe	5'-CCC GCG TCA GAG CCA CAG TCC T-3'
HO-1	sense	5'-CAC AGG GTG ACA GAA GAG GCT AA-3'
	antisense	5'-CTG GTC TTT GTG TTC CTC TGT CAG-3'
	probe	5'-CAG CTC CTC AAA CAG CTC AAT GTT GAG C-3'
αSMA	sense	5'-GCC AGT CGC CAT CAG GAA C-3'
	antisense	5'-CAC ACC AGA GCT GTG CTG TCT T-3'
	probe	5'-CTT CAC ACA TAG CTG GAG CAG CTT CTC GA-3'
Bsep	sense	5'-CCA AGC TGC CAA GGA TGC TA-3'
	antisense	5'-CCT TCT CCA ACA AGG GTG TCA-3'
	probe	5'-CAT TAT GGC CCT GCC GCA GCA-3'

Double fluorescence immunostaining

Double staining was performed with a sequential fluorescent method on 20 μm-thick rat liver cryostat sections. The cryostat sections were dried overnight at room temperature and subsequently fixed in acetone for 10 minutes and finally washed in PBS, immediately before

use. Then, the sections were incubated with a mixture of normal rabbit serum and normal swine serum, diluted 1:5 in PBS for 30 minutes.
Sections were incubated with primary antibodies against the ABC transporters: Mrpl, Mrp3, and C219 (pan-P-glycoproteins) and with primary antibodies against HSC markers: desmin and GFAP at room temperature for 45 min., followed by tetramethylrhodamine isothiocyanate (TRITC)-labeled swine anti-rabbit antibodies (Dako, Glostrup, Denmark) and finally, followed by fluoresceine isothiocyanate (FITC)-labeled rabbit anti-mouse antibodies (DAKO). The antibodies were diluted in PBS. All incubation steps were followed by a wash in three changes of PBS. Sections were mounted with medium containing para-phenylene-diamine. Controls consisted of omission of either primary or one of the secondary antibodies and reversal of the order of incubation with antibodies. No aspecific labelling could be detected. Double staining was detected using confocal laser scanning microscopy (Zeiss 410 inverted laser scan microscope).

Table 2. Sequences of human PCR primers and probes used for real-time detection PCR analysis

Gene		Primers
18S	sense	5'-CGG CTA CCA CAT CCA AGG A-3'
	antisense	5'-CCA ATT ACA GGG CCT CGA AA-3'
	probe	5'-CGC GCA AAT TAC CCA CTC CCG A-3'
MRPI	sense	5'-GGT GGG CCG AGT GGA ATT-3'
	antisense	5'-TTG ATG TGC CTG AGA ACG AAG T-3'
	probe	5'-CTG CCT GCG CTA CCG AGA GGA CCT-3'
MRP2	sense	5'-TGC AGC CTC CAT AAC CAT GAG-3'
	antisense	5'-CTT CGT CTT CCT TCA GGC TAT TCA-3'
	probe	5'-CAG CTT TCG TCG AAC ACT TAG CCG CA-3'
MRP3	sense	5'-GCC ATC GAC CTG GAG ACT GA-3'
	antisense	5'-GAC CCT GGT GTA GTC CAT GAT AGT G-3'
	probe	5'-CAT CCG CAC CCA GTT TGA TAC CTG CAC-3'
MRP4	sense	5'-AAG TGA ACA ACC TCC AGT TCC AG-3'
	antisense	5'-GGC TCT CCA GAG CAC CAT CT-3'
	probe	5'-CAA ACC GAA GAC TCT GAG AAG GTA CGA TTC CT-3'
MRP5	sense	5'-TGA AAG CCA TTC GAG GAG TTG-3'
	antisense	5'-CGG AAA AGC TCG TCA TGC A-3'
	probe	5'-CTC GCA GCG TGC CCT TGA CAA AG-3'
MRP6	sense	5'-AGA CAC GGT TGA CGT GGA CAT-3'
	antisense	5'-GCT GAC CTC CAG GAG TCC AA-3'
	probe	5'-CCA GAC AAA CTC CGG TCC CTG CTG AT-3'
MDR1	sense	5'-GGC AAA GAA ATA AAG CGA CTG AA-3'
	antisense	5'-GGC TGT TGT CTC CAT AGG CAA T-3'
	probe	5'-CGT GTC CCA GGA GCC CAT CCT GT-3'
BSEP	sense	5'-ACA TGC TTG CGA GGA CCT TTA-3'
	antisense	5'-GGA GGT TCG TGC ACC AGG TA-3'
	probe	5'-CCA TCC GGC AAC GCT CCA AGT CT-3'

Isolation of nuclear extracts
HSCs were washed twice with ice cold PBS and harvested in 500 µl PBS. Nuclear extracts were isolated as described previously, using 0.25% Nonidet P-40 (25).

Electrophoretic mobility shift assay (EMSA)
EMSAs were performed as described previously (25) with 5 micrograms of nuclear proteins in a total volume of 15 µl. The mdr-κB probe used, 5'-gat cctggg gaa ttc cag ctc-3' with the NF-κB site underlined has been published before (26). Competition was performed using excess nonlabeled radioactive oligonucleotide. The reaction mixture was analyzed on a 4% polyacrylamide gel.

Immunocytochemistry
Analysis of active caspase-3 and α-smooth muscle actin (α-SMA) were performed on cells cultured on coverslips. Coverslips were washed in PBS and fixed in 4% paraformaldehyde for 10 minutes. Coverslips were then stored in 0.1% PBS at 4°C. Prior to staining, cells were washed in PBS, followed by incubation in PBS containing 1% Triton-X100 for 5 minutes and washed twice with PBS. Primary antibodies against active caspase-3 (rabbit polyclonal antibody from Cell Signaling Technology, Beverly, MA, USA) and α-SMA (mouse monoclonal antibody from Sigma Aldrich) were used at a dilution of 1:100 and 1:1000 respectively in 0.5% BSA/PBS for 2 hours. Horseradish peroxidase-conjugated goat-anti-rabbit Ig (fluorescent conjugate, Invitrogen) and horseradish peroxidase-conjugated goat-anti-mouse Ig (fluorescent conjugate, Invitrogen) were used as secondary antibodies at a dilution of 1:400 for 1 hour. Coverslips were subsequently washed with PBS and mounted in fluorescent mounting medium S3023 (DAKO). All slides were evaluated on a Leica confocal laser-scanning microscope.

Flow cytometry, transporter efflux assay
HSCs were incubated with either 200 ng/mL rhodamine 123 (Molecular Probes) as a Mdr-substrate or 0.1 μmol/L Cell TrackerTM Green CMFDA (Invitrogen) as a Mrp-substrate for 30 minutes, with and without their corresponding Mdr and Mrp inhibitors. Cells were washed and exposed to either Mdr and Mrp inhibitors without Mdr/Mrp substrate for another 60 minutes. Cells were then trypsinized, harvested, and incubated for 15 minutes in ice cold PBS. After incubation, 5000 cells per sample were counted and viable cells were analysed for rhodamine 123 or CMFDA fluorescence using a FACS Calibur (BD Bioscience, Alphen aan de Rijn, the Netherlands).

Acridine orange and Sytox green nuclear staining
Cells were seeded in 12-well plates and treated as indicated. Apoptosis was shown by determining nuclear condensation assessed by acridine orange staining at 2.5 μg/mL (Sigma Aldrich). Necrosis was shown by Sytox Green nucleic acid staining at 0.5 μmol/L (Invitrogen). Cells were monitored over time using a Olympus CKX41 microscope.

Statistical analysis
Statistical analysis was performed using SPSS software, version 12.0. Results are expressed as the mean value and standard deviation. Statistical significance was calculated using a Student's t test, or a one-way ANOVA with Bonferroni post-hoc analysis for correction for multiple comparison. A corrected p value <0.05 was considered to be statistically significant.

Results

Activated rat HSCs have a different ABC transporter mRNA profile from hepatocytes
To determine whether activated rat HSCs express ABC transporters, we isolated mRNA from these cells and analyzed the expression of selected ABC transporter genes. We compared this to the ABC transporter expression profile of freshly isolated rat hepatocytes (Figure 1B/C). Rat hepatocytes highly expressed ABC transporters that are active in bile formation, including Mrp2 and Bsep. As expected, the expression of these genes was low to undetectable in activated HSCs. In contrast, the expression of Mrp1 was particularly high in activated HSCs and this expression was clearly associated with the activated phenotype, as Mrp1 expression increased upon activation of quiescent stellate cells (Figure 1D). In addition, comparable expression levels

of Mdr1a, Mdr1b, Mrp3 and Mrp4 were observed in activated HSCs and hepatocytes. Expression of Mrp5 remained undetectable in both cell types (Figure 1B/C).

The MRP transporter profile of rat HSCs is similar to human HSCs

MRP1 was the most prominent transporter in human HSCs, as well as in rat HSCs (Figure 1A/B). Expression levels of MRP3/Mrp3 and MRP4/Mrp4 were also similar in rat and human cells. However, MRP5 was expressed in human HSCs, whereas we were unable to detect Mrp5 in rat HSCs. MDR1 expression in human HSCs was low compared to rat HSCs.

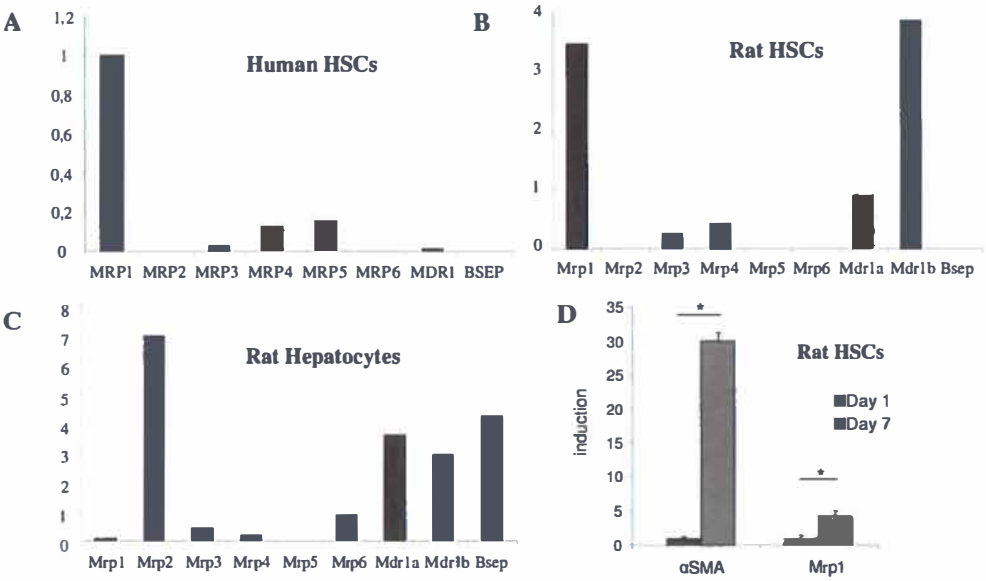

Figure 1. Activated human and rat HSCs show high expression of Mrp1. A-C) Relative mRNA expression levels were normalized to 18S. The human MRP1 δδCt-value was set to 1, for other genes and cell types, the δδCt-values were compared to human MRP1. Data are expressed as means ± SD. MRP1/Mrp1 mRNA was abundant in activated HSCs, while MRP2/Mrp2, MRP6/Mrp6 and BSEP/Bsep were undetectable in HSCs but present in hepatocytes. Mrp5 was absent in rat HSCs and in hepatocytes, but not in human HSCs. Mrp3 and Mrp4 levels were comparable in HSCs and hepatocytes. Mdr1a and Mdr1b were prominently expressed in rat HSCs and hepatocytes. However, MDR1 expression in human HSCs was low. B) Freshly harvested hepatic stellate cells were cultured on plastic for 1 and 7 days. As expected, stellate cell activation was associated with induction of αSMA mRNA expression ($p<0,05$). Expression of Mrp1 also increased 4-fold upon activation of quiescent stellate cells ($p<0,05$).

Mrp1 is present in HSCs in experimental fibrosis in rats

Liver slices from rats exposed to CCl4 for 2 weeks were stained for Mdr-transporters, Mrp1 and Mrp3. Desmin and GFAP were used as markers for HSCs. Desmin positive cells were also positive for Mrp1 (Figure 2C). However, no overlap was found between desmin or GFAP with Mdr-transporters or Mrp3 (data not shown).

Cytokines induce Mdr1b mRNA levels in activated HSCs

Expression of several ABC transporters has been shown to be regulated during inflammation and/or oxidative stress. Therefore, we investigated whether cytokines may influenced the expression level of the most prominent ABC-transporters present in HSCs. Activated HSCs were exposed for 6 hours to a cytokine mixture (CM) consisting of 20 ng/mL mTNF-α, 10 ng/mL hIL-1β and 10 ng/mL rIFN-γ. Total RNA was isolated. mRNA levels of the NF-κB-controlled gene inducible

nitric oxide synthase (iNOS) was significantly increased in the CM-treated HSC cells (>1,000-fold; Figure 3A). Of the ABC transporter genes tested, only Mdr1b expression was significantly induced by CM treatment (4-fold; Figure 3B/C).

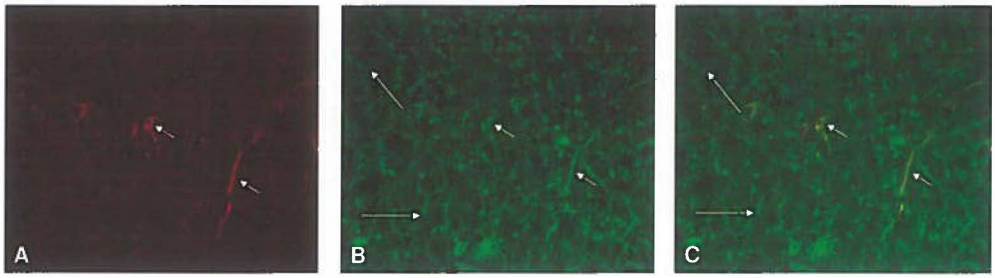

Figure 2. HSCs express Mrp1 in CCl4 treated rats. Double immunofluorescence staining for desmin, a marker of HSCs (A) and Mrp1 (C) in rats treated with CCl4 for two weeks. A) desmin positive HSCs (red labelling, short arrow). B) Mrp1 positive HSC (green labelling, short arrow) and a specific focal membranous staining pattern of Mrp1 in hepatocytes (green labelling, long arrow). C) Merged image: desmin positive HSC cells are also Mrp1 positive (yellow labelling, short arrow). In addition, Mrp1 was also expressed in the basolateral membrane of hepatocytes, serving as internal positive control (only green labelling, long arrow).

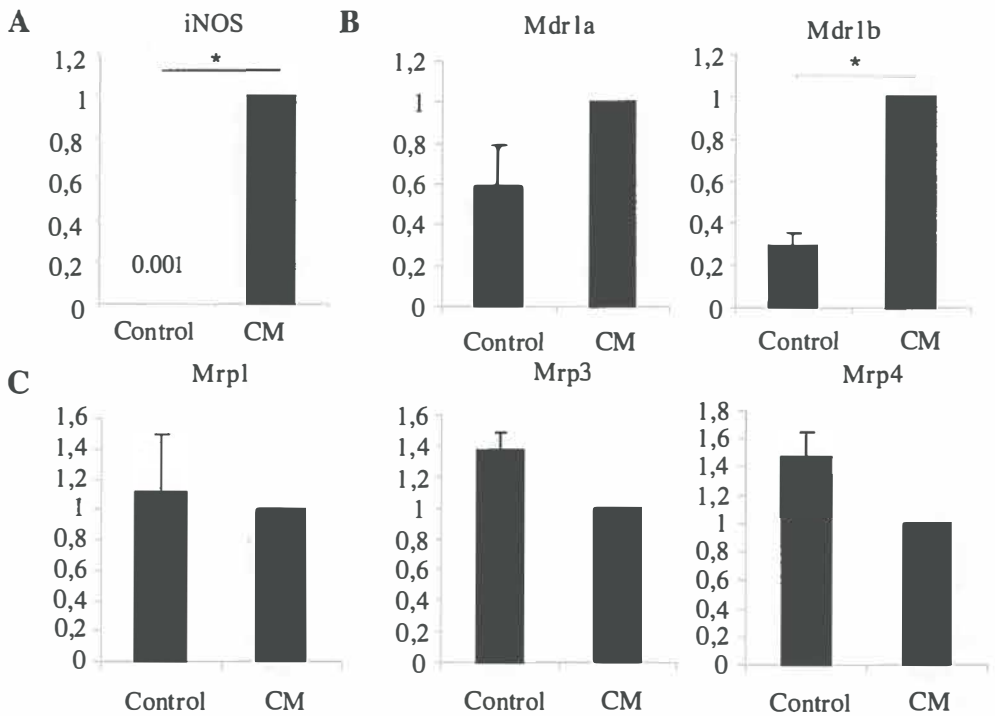

Figure 3. Mdr1b is a cytokine inducible NF-κB responsive gene. Cytokine mixture (CM: TNF-α 20 ng/mL, IL-1β 10 ng/mL, IFN-γ 10 ng/mL) significantly induced the NF-κB responsive gene inducible nitric oxide synthase (iNOS), which was abolished by Ad5IκBAA. Mdr1b, like iNOS, was significantly induced by cytokines, unlike Mdr1a and Mrp-type transporters. Cells exposed to Ad5LacZ and Ad5IκB were incubated with virus at an MOI of 75 for 24 hours prior to the experiment. A) Quantitative real-time PCR for iNOS, the numbers above the left bar represent the fold induction compared to CM treated cells (* $p<0,05$). B) Quantitative real-time PCR for Mdr1a and Mdr1b (* $p<0,05$). C) Quantitative real-time PCR for Mrp1, Mrp3 and Mrp4 (* $p<0,05$).

Mdr1b induction by cytokines is NF-κB dependent in activated HSCs

Next, we determined whether cytokine-induced Mdr1b expression was dependent on NF-κB activation. Nuclei were isolated from activated HSCs that had been exposed to CM for 6 hrs as well as untreated HSCs. EMSA analyses using these nuclear extracts and an NF-κB-specific DNA probe showed that the nuclei of CM-treated HSCs contain NF-κB, while this transcription factor was absent in control nuclei (Figure 4A). Co-treatment with a dominant negative NF-κB adenovirus (Ad5IκBAA) abolishesed both the level of nuclear NF-κB (Figure 4A) and the CM-dependent Mdr1b expression in CM-treated HSCs (Figure 4B). Infection with Ad5LacZ did not decrease nuclear NF-κB levels nor Mdr1b mRNA expression levels (Figure 4A/B). The effect of CM-treatment and NF-κB inhibition on Mdr1b expression was paralleled by iNOS, a well known target gene of NF-κB (Figure 4B).

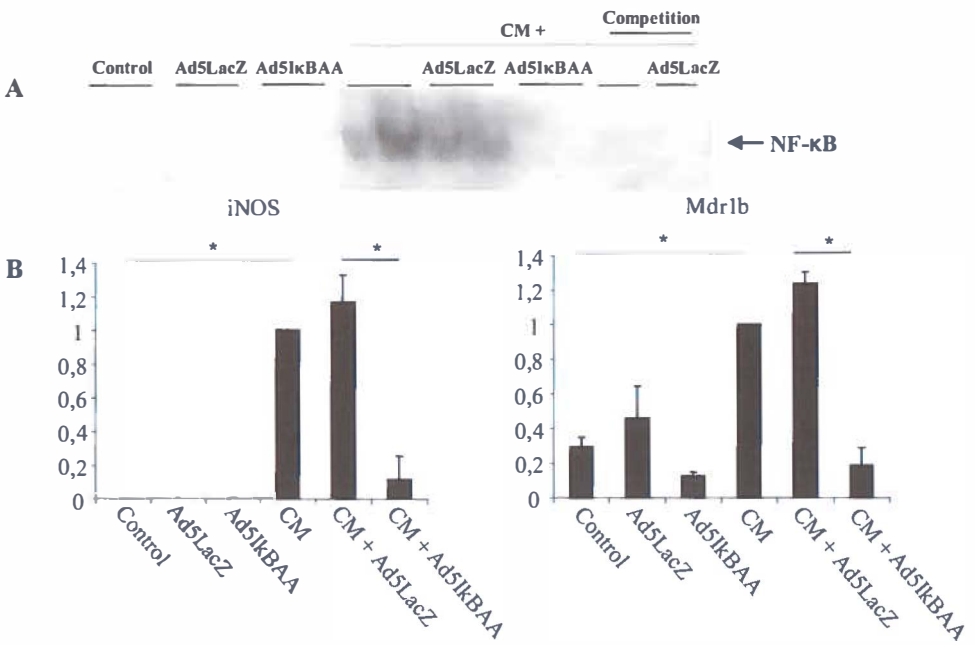

Figure 4. NF-κB is not activated in HSCs but is activated by cytokines and iNOS and Mdr1b are NF-κB responsive genes. A) EMSA for NF-κB. In untreated activated hepatic stellate cells, NF-κB was not activated. Cells exposed to a cytokine mixture (TNF-α 20 ng/mL, IL-1β 10 ng/mL, IFN-γ 10 ng/mL) for 6 hrs demonstrated activation of NF-κB, which was abolished by the dominant negative NF-κB adenovirus. Competition with excess nonlabeled oligonucleotide completely abolished CM induced NF-κB signaling. B) Quantitative real-time PCR, showing significant reduction in iNOS and Mdr1b expression when NF-κB is inhibited (* $p<0,05$).

Oxidative stress is not a major regulator of Mrp and Mdr mRNA expression in activated HSCs

In order to determine whether other disease conditions affect the ABC transporter expression in HSCs, we studied the effect of different inducers of oxidative stress on their transcription levels. HSCs were exposed for 6 hours to either 200 μmol/L hydrogen peroxide (H_2O_2), or 10 μmol/L menadione (an intracellular generator of superoxide anions). Under these conditions, expression of a typical marker gene for oxidative stress, HO-1, was significantly induced, albeit at variable levels (Figure 5A). In contrast, only Mdr1a was slightly induced with hydrogen peroxide (Figure 5B). Mdr1b and Mrp transporters were not significantly altered by exposure to exogenous reactive oxygen species (Figure 5B/C).

Both Mdr-type and Mrp-type transporters are functional efflux transporters in activated HSCs
To determine whether Mdr transporters are not only present, but also function as efflux transporters, we used a flow cytometry efflux assay. We exposed cells to the Mdr-substrate rhodamine 123, with or without 50 µmol/L verapamil or 50 µmol/L PSC-833 as Mdr-inhibitors. Cells incubated with both rhodamine 123 and either one of the Mdr-inhibitors showed significantly higher fluorescence content than cells incubated with rhodamine 123 alone (Figure 6A). This data demonstrates that the Mdr-transporters that are present, also function as efflux transporters, and shows that both verapamil and PSC-833 are efficient Mdr-inhibitors.

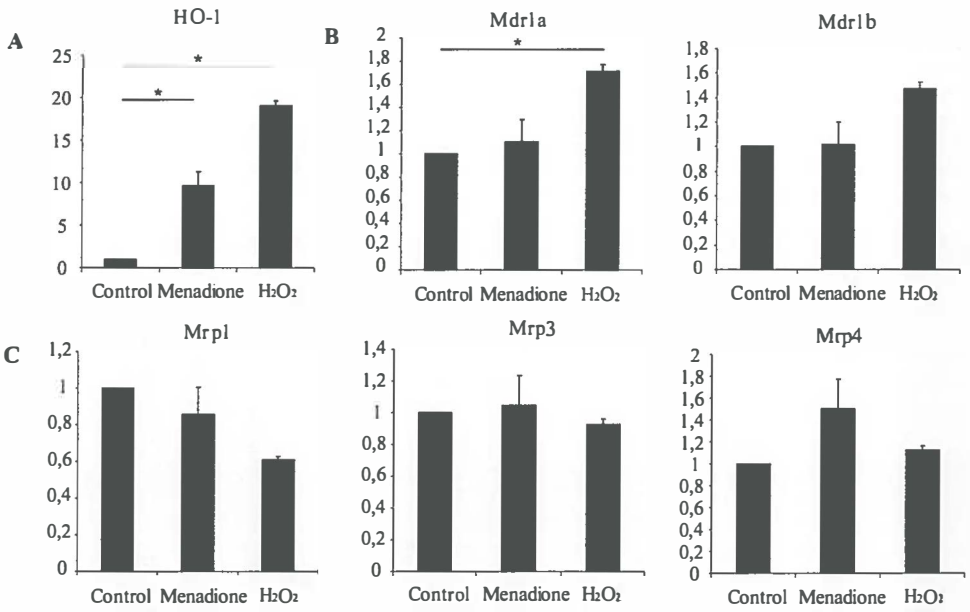

Figure 5. Oxidative stress is not a major regulator of ABC transporter expression. The oxidative stress responsive gene heme-oxygenase 1 (HO-1) was significantly induced by 10 µmol/L menadione or 200 µmol/L H_2O_2. Mdr1a expression was slightly increased by hydrogen peroxide, whereas other Mrp and Mdr transporters were not significantly altered by exposure to reactive oxygen species. A) Quantitative real-time PCR for HO-1 (* $p<0,05$). B) Quantitative real-time PCR for Mdr1a and Mdr1b (* $p<0,05$). C) Quantitative realtime PCR for Mrp1, Mrp3 and Mrp4 (* $p<0,05$).

To determine whether Mrp transporters are not only present, but also function as efflux transporters, we used a flow cytometry efflux essay. We exposed cells to the Mrp-substrate CMFDA, with and without 50 µmol/L MK571 as Mrp-inhibitor. Cells incubated with both CMFDA and MK571 showed a significantly higher fluorescence content than cells incubated with MK571 alone (Figure 6B). This demonstrates that the Mrp-transporters that are present, also function as efflux transporters, and shows that MK571 is an efficient Mrp-inhibitor.

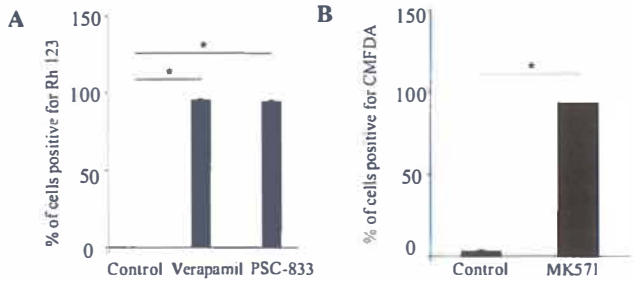

Figure 6. Mdr and Mrp transporters are functional efflux transporters in activated hepatic stellate cells. A) Cells were exposed to 200 ng/mL rhodamine 123 with or without either 50 µmol/L verapamil or 50 µmol/L PSC-833. 95.5% and 94.2% of cells exposed to rhodamine 123 and verapamil or PSC-833 respectively were considered positive for rhodamine 123 fluorescence, versus 0.2% of cells exposed to rhodamine 123 alone (* $p<0.05$). B) Cells were exposed to 0.1 µmol/L CMFDA with or without 50 µmol/L MK571. 92.4% of cells exposed to CMFDA and MK571 was considered positive for CMFDA fluorescence, versus 3.0% of cells exposed to CMFDA alone. Only viable cells were included in the analysis (* $p<0.05$).

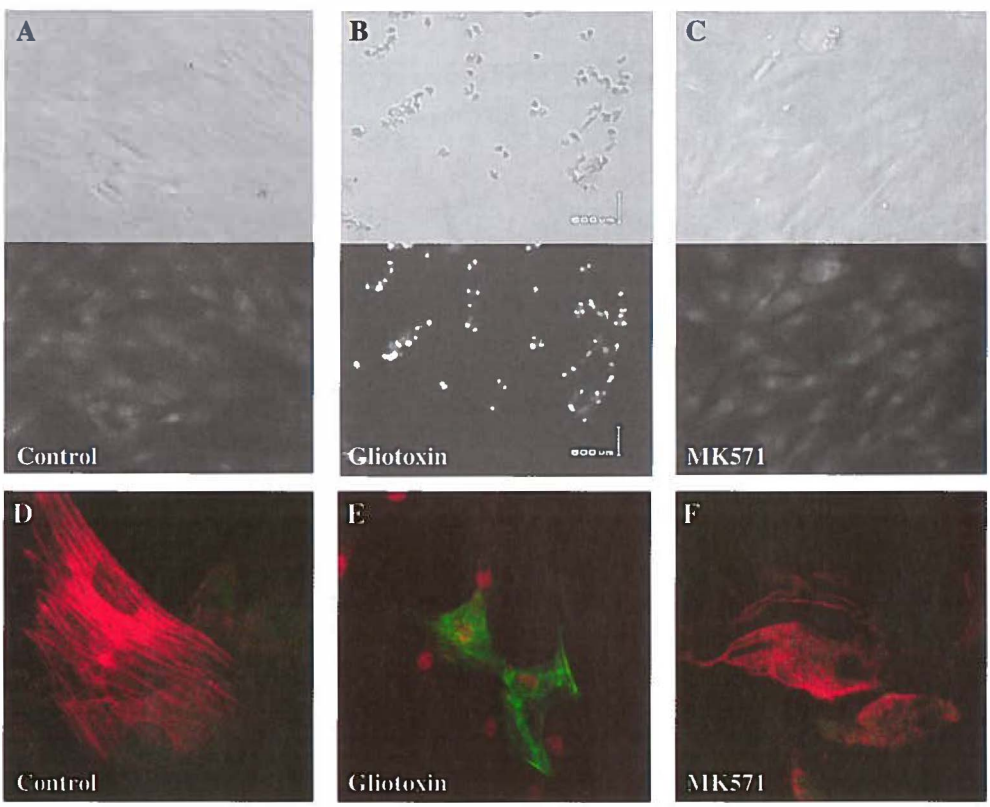

Figure 7. MK571 does not cause apoptosis in activated hepatic stellate cells. Cells were exposed to 50 µmol/L MK571 for 6 hours. At concentrations of 50 µmol/L, MK571 did not induce apoptosis as judged by nuclear morphology with acridine orange staining (C) and a lack of activated caspase-3 staining (F, green), but cell morphology was changed in cells treated with MK571 for 6 hours as judged by a staining for α-smooth muscle actin (F, red). Gliotoxin was used as a positive control for HSC apoptosis (B/E). The disruption of the actin cytoskeleton (red) and staining of active caspase-3 (green) as a marker for apoptosis are shown in panel E. Panel A and D depict untreated cells.

Mrp-type transporters are essential for the viability of activated HSCs

In order to determine the function of ABC transporter expression in activated HSCs, we used various inhibitors of ABC transporters and evaluated their effects on HSC viability. First, we tested whether inhibition of Mrp function by MK571 induced apoptosis of activated HSCs (Figure 7, red: actin cytoskeleton, green: active caspase-3). Gliotoxin, which is known to induce HSC apoptosis, was used as a positive control. Indeed, gliotoxin induced typical characteristics of apoptosis in HSCs, such as condensed nuclei (Figure 7B) and activation of caspase-3 (Figure 7E), which was not observed in untreated cells (Figure 7A and D). In contrast, MK571, a selective inhibitor of Mrp function, does not cause nuclear condensation (Figure 7C) or activation of caspase-3 (Figure 7F). However, MK571 treatment clearly affected HSC cell morphology and disrupted the α-smooth muscle actin cytoskeleton (Figure 7F). Therefore, we tested whether MK571 induces HSC necrosis. Indeed, over 50% of the HSCs became necrotic, as early as 6 hours after exposure to 50 μmol/L MK571 (Figure 8B). After an additional 18 hours, no viable HSCs were detected. All cells contained Sytox green fluorescent nuclei, indicative for necrotic cell death (Figure 8C). In parallel experiments, 50 μmol/L MK571 did not significantly affect hepatocyte viability (data not shown). The Mdr-inhibitors verapamil (50 μmol/L) and PSC-833 (50 μmol/L) caused neither apoptotic nor necrotic death of activated HSCs (Figure9A-C).

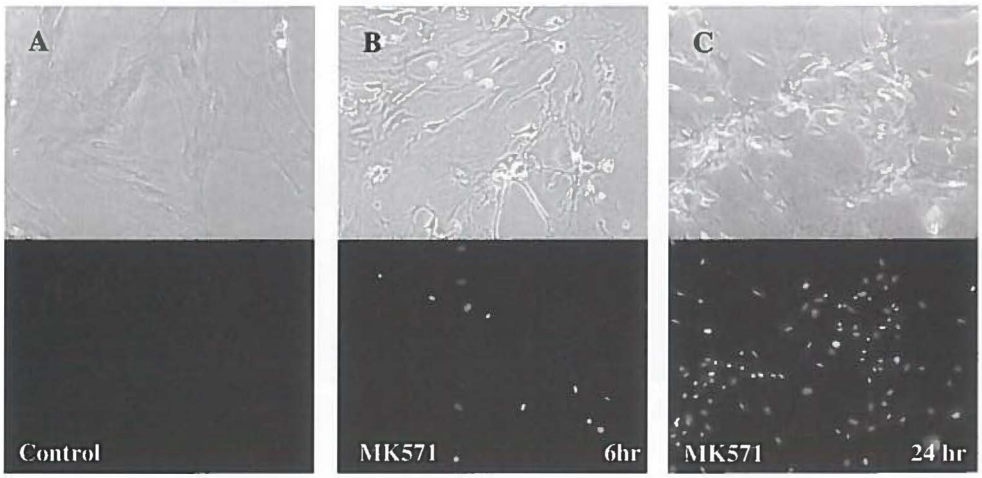

Figure 8. MK571 does cause necrosis in activated hepatic stellate cells. Cells were exposed to 50 μmol/L MK571 for the indicated time. Sytox green staining, indicative for necrotic cell death, clearly demonstrated that inhibition of Mrp-type transportes induced necrotic cell death in activated stellate cells (B/C). Panel A depicts untreated cells.

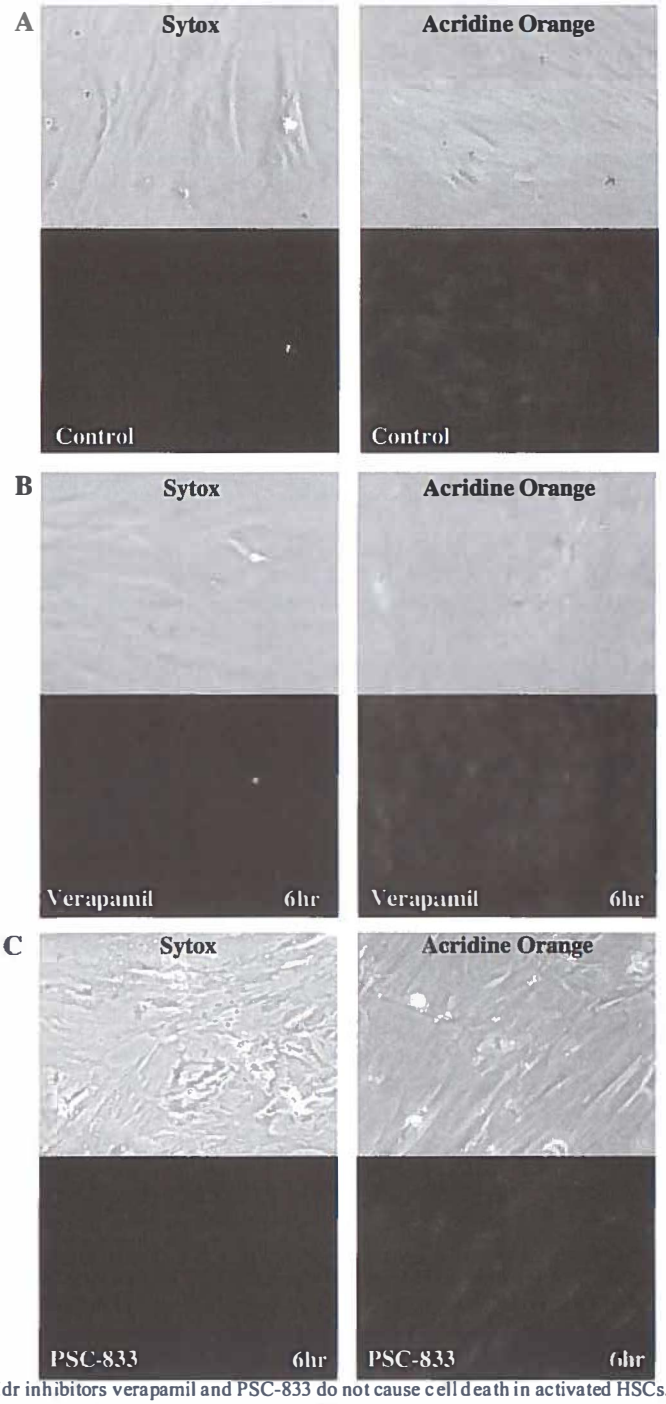

Figure 9. The Mdr inhibitors verapamil and PSC-833 do not cause cell death in activated HSCs. Cells were untreated (A) or exposed to 50 µmol/L verapamil (B) or 50 µmol/L PSC-833 (C) for 6 hours. Neither verapamil nor PSC-833 induced necrosis as judged by Sytox green nuclear staining, or apoptosis as judged by nuclear morphology using acridine orange staining.

Discussion

While the function of many ABC transporters has been studied extensively in hepatocytes (12) and hepatic progenitor cells (14), their presence and function in activated hepatic stellate cells has never been investigated. In this study we showed that activated rat HSCs expressed Mrp1, Mrp3, Mrp4, Mdr1a and Mdr1b mRNA. This is a similar expression pattern as seen in hepatic progenitor cells (14, 27). Activated human HSCs also expressed MRP1, MRP3 and MRP4 and, unlike rat HSCs, also MRP5. Expression of MDR1 in human HSCs was low compared to rat HSCs. Both human and rat HSCs did not express Mrp2/MRP2, Mrp6/MRP6 and Bsep/BSEP, which are prominent transporters in rat hepatocytes. Compared to rat hepatocytes, Mrp1 expression was high in both rat and human HSCs. In immunohistochemical double staining of liver sections from rats treated with carbon tetrachloride for 2 weeks, desmin positive HSCs also stained for Mrp1. However, no overlap could be found between desmin or GFAP with Mrp3 or Mdr-transporters.

High expression of Mrp-type and Mdr-type transporters is associated with enhanced resistance to cell death (11). Activated and proliferating HSCs may use these transporters to clear their cells of exogenous and endogenous toxic substrates generated in the injured liver, endowing these cells with a survival advantage in a hostile environment. We showed that inhibition of Mrp-type transporters with MK571 induced cell death in activated HSCs, which could be caused by accumulation of a toxic endogenous Mrp substrate. As reported for other cell types (28, 29), the Mdr1b mRNA level in activated stellate cells was increased by cytokines in a NF-κB-dependent manner. No significant changes were observed in Mrp1, Mrp3 and Mrp4 mRNA levels of activated stellate cells exposed to cytokines. The Mrp regulatory response to cytokines varies between cell types. Lee and Piquette-Miller describe an increase in Mrp3 expression in Huh7 cells in response to cytokine exposure. TNF-α treated HepG2 cells showed a decrease in expression of Mrp1 and Mrp3, while Mrp1 could be induced with IL-1β (30). We used a cytokine mixture to mimic the cytokine profile observed in inflammatory conditions (31). Oxidative stress did not change the expression of Mrp-type ABC transporters, and hydrogen peroxide only slightly increased Mdr1a expression. Little is known about the regulation of Mrp- and Mdr-type transporters by oxidative stress. Our results could imply that reactive oxygen species, at least in stellate cells, are not major regulators of these transporters. Stellate cells do respond to reactive oxygen species as demonstrated by the increased expression of the oxidative stress-responsive gene heme oxygenase-1 (HO-1). Yet, there could be a role for Mrp-type transporters in the detoxification of reactive oxygen species. The expression of Mrp-type transporters in stellate cells was significant and these transporters are known to be involved in the efflux of glutathione-conjugates, generated in the detoxification of reactive oxygen species. Furthermore, Hammond et al. reported on Mrp1 dependent efflux of reduced glutathione in several cell types (32). According to this study, Mrp1-dependent efflux of reduced glutathione is a prerequisite for apoptosis. The interrelationship between reactive oxygen species, glutathione status and cell death is currently investigated in our laboratory.

Compared to activated stellate cells, Mrp1 mRNA expression is low in hepatocytes. However, hepatocytes express other Mrp-type efflux pumps, e.g. Mrp3 and Mrp2. The latter is hepatocyte-specific in the liver and localized at the canalicular membrane and plays an important role in bile formation. The differential expression and/or localization of Mrp-type transporters in hepatocytes and activated stellate cells, may explain the differential sensitivity to inhibition of Mrp-function.

Although culture-activated rat HSCs expressed Mdr-transporters that were functional as efflux transporters, we could not detect Mdr-transporters in immunohistochemical double staining in

liver sections of carbon tetrachloride treated rats. Also, the Mdr inhibitors verapamil and PSC-833 (cyclosporin A analogue) did not affect cell death in culture-activated HSCs. Although Mdr-transporters did not seem as important as Mrp-transporters for the survival of activated hepatic stellate cells in our in vitro conditions, they might play a role in vivo by transporting exogenous toxins or xenobiotics.

In conclusion, these results demonstrate that activated hepatic stellate cells express high levels of several Mrp-type and Mdr-type transporters in conditions prevailing in the chronically injured liver, which may enable these cells to survive in this hostile environment. We hypothesize that MK571 induces cell death in HSCs by inhibiting Mrp1. Although the Mrp substrate involved in this process has not been elucidated, we suggest that inhibiting Mrp1 function in chronic liver disease may cause cell death in activated HSCs, without affecting hepatocytes, thereby presenting an interesting target for drug therapy aimed at preventing or reversing liver fibrosis and cirrhosis. Since activation and proliferation of hepatic stellate cells is the common denominator of most chronic liver diseases, this strategy holds great promise for the treatment of chronic liver diseases, regardless of the etiology (6).

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Chapter 3

Superoxide anions and hydrogen peroxide inhibit proliferation of activated rat stellate cells and induce different modes of cell death

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Submitted

Abstract

Background
In chronic liver injury, hepatic stellate cells (HSCs) proliferate and produce excessive amounts of connective tissue causing liver fibrosis and cirrhosis. Oxidative stress has been implicated as a driving force of HSC activation and proliferation, although contradictory results have been described.

Aim
To determine the effects of oxidative stress on activated HSC proliferation, survival and signaling pathways.

Methods
Serum-starved culture-activated rat HSCs were exposed to the superoxide anion donor menadione (5-25 μmol/L) or hydrogen peroxide (0.2-5 mmol/L). Heme oxygenase-1 mRNA expression, glutathione status, cell death, phosphorylation of MAP kinases and proliferation were investigated.

Results
Menadione induced apoptosis in a dose- and time-dependent, but caspase-independent manner. Hydrogen peroxide induced necrosis only at extremely high concentrations. Both menadione and hydrogen peroxide activated JNK and p38. Hydrogen peroxide also activated ERK. Menadione, but not hydrogen peroxide, reduced cellular glutathione levels. Inhibition of JNK or supplementation of glutathione reduced menadione-induced apoptosis. Nontoxic menadione and hydrogen peroxide inhibited PDGF- or/and serum-induced proliferation.

Conclusion
Reactive oxygen species inhibit HSC proliferation and promote HSC cell death in vitro. Different reactive oxygen species induce different modes of cell death. Superoxide anion-induced HSC apoptosis is dependent on JNK activation and glutathione status.

Introduction

Chronic liver diseases almost invariably result in fibrosis leading to end-stage cirrhosis. At present, no effective drug-based therapy exists for liver fibrosis or cirrhosis and liver transplantation is the only life-saving therapy. However, liver transplantation programs are frustrated by organ shortage, peri- and postoperative morbidity, rejection and the long-term use of immunosuppressants. Liver fibrosis and cirrhosis are characterized by the formation of scar tissue and impaired liver function due to proliferation and activation of extracellular matrix-producing hepatic stellate cells (HSCs) and progressive loss of hepatocytes. During activation, the HSCs lose their retinoid-storing capacity and adopt a myofibroblast-like phenotype (*1*).
In chronic liver diseases, all liver cells are exposed to increased levels of bile acids, inflammatory cytokines and oxidative stress. Paradoxically, the hepatocytes perish under these pathophysiological conditions, whereas the activated HSCs florish. Previously, we have reported on the differential response to bile acids between hepatocytes and activated HSCs (2, 3). In addition, we have reported on the cytotoxic effects of reactive oxygen species (ROS) on hepatocytes (4, 5). Although oxidative stress has been implicated in stellate cell activation and fibrogenesis, the effects of reactive oxygen species on HSCs remains unclear, since contradictory results have been reported: both mitogenic (6-12) and cell-death inducing properties (13-15). Part of the conflicting results may be due to the use of different sources of ROS and the lack of an integrated approach, where both proliferation and cell death are analyzed in the same experimental setting.
Therefore, the aim of this study is to investigate the effect of two different kinds of reactive oxygen species, superoxide anions and hydrogen peroxide, at concentrations found in the literature (5, 15, 16), on HSC proliferation, metabolic activity, and cell death.

Materials and methods

Animals
Specified pathogen-free male Wistar rats were purchased from Harlan (Zeist, The Netherlands). They were housed under standard laboratory conditions with free access to standard laboratory chow and water. All experiments were performed following the guidelines of the local committee for care and use of laboratory animals.

Hepatic stellate cell isolation and culture
Hepatic stellate cells (HSCs) were isolated from male Wistar rats (500-600 g) by pronase (Merck, Amsterdam, the Netherlands) and collagenase-P (Roche, Almere, The Netherlands) perfusion of the liver, followed by Nycodenz (Axis-Shield POC, Oslo, Norway) gradient (12% w/v) centrifugation as described previously (17). Cells were then cultured in Iscove's Modified Dulbecco's Medium with Glutamax (Invitrogen, Breda, The Netherlands) supplemented with 20% heat-inactivated fetal calf serum (Invitrogen), 1 mmol/L sodium-pyruvate (Invitrogen), 1x MEM non essential amino acids (Invitrogen), 50 µg/mL gentamycin (Invitrogen), 100 U/mL penicillin (Lonza, Vervier, Belgium), 10 µg/mL streptomycin (Lonza), 250 ng/mL fungizone (Lonza) and 250 U/mL Nystatin (Sanofi-Synthelabo, Maassluis, The Netherlands) in a humidified atmosphere containing 5% CO2 at 37 °C. Primary HSC cultures were allowed to grow to confluence, passaged via trypsinization, and then cultured in Iscove's medium with supplements as described above, except Nystatin. Prior to experiments, HSCs were serum-starved for 24 hours, unless indicated otherwise.

Experimental design
Culture activated rat HSCs (passage 3 or 4) were exposed to oxidative stress induced by 5–25 µmol/L of the superoxide anion donor menadione (2-Methyl-1,4-naphthoquinone, Sigma-Aldrich, Zwijndrecht, The Netherlands) or 0.2–5 mmol/L hydrogen peroxide (Merck). Menadione generates superoxide anions intracellularly via a continuous cycle of reduction-oxidation reactions (18). The JNK inhibitor SP600125 (Calbiochem, San Diego, USA), the p38 inhibitor SB-203850 (Calbiochem) and the ERK inhibitor U0126 (Promega, Leiden, The Netherlands) were all used at 10 µmol/L. The cell permeable glutathione donor GSH-monoethylester (GSH-MEE, Calbiochem) was used at 5 mmol/L. Catalase and superoxide dismutase conjugated to polyethylene glycol (PEG-catalase and PEG-SOD, Sigma-Aldrich) were used at 500 U/mL and 200 U/mL respectively. Platelet-derived growth factor (PDGF: R & D Systems, Abingdon, UK) was used at 10 ng/mL. The caspase-3 inhibitor (Z-DEVD-FMK: R & D Systems) was used at 0.05 µmol/L, the caspase-6 inhibitor (Z-VEID-FMK: R & D Systems) and the caspase-9 inhibitor (Z-LEHD-FMK: R & D Systems) were used at 0.2 µmol/L. Inhibitors were added 30 minutes prior to exposure to menadione or hydrogen peroxide.

RNA isolation
RNA was isolated using Tri-reagent (Sigma-Aldrich) according to the manufacturer's instructions. Reverse transcription was performed on total RNA using random nonamers (Sigma-Aldrich) in a final volume of 50 µl. Reverse transcription was performed in three steps: 10 minutes at 25°C, 1 hour at 37°C and 5 minutes at 95°C.

Quantitative Real-Time PCR
Real time detection was performed on the ABI PRISM 7700 (PE Applied Biosystems) initialized by 10 min at 95 °C, followed by 40 cycles (15 seconds at 95 °C, and 1 minute at 60 °C). Each sample was analyzed in duplicate. mRNA levels of 18S were used as an internal control. Reaction mixture contained qPCR mastermix plus-dTTP (Eurogentec, Maastricht, The Netherlands) supplemented with 900 nmol/L sense and anti-sense primer and 200 nmol/L labeled probe. The following primers (invitrogen) and probe (eurogentec) were used for 18s: sense primer 5'-cgg cta cca cat cca agg a-3'; antisense primer 5'-cca att aca ggg cct cga aa-3'; probe 5'-cgc gca aat tac cca ctc ccg a-3', based on gen bank m11188. For HO-1 mRNA detection, the following primers (invitrogen) and probe (eurogentec) were used: sense primer 5'-cac agg gtg aca gaa gag gct aa-3'; antisense primer 5'-ctg gtc ttt gtg ttc ctc tgt cag-3'; probe 5'-cag ctc ctc aaa cag ctc aat gtt gag c-3', based on Gen Bank NM012580.

Glutathione assay
Glutathione and glutathione disulfide content was determined using a spectrophotometry based assay as described previously (19). Samples for glutathione determination were harvested in cell lysis buffer composed of 25 mmol/L HEPES, 5 mmol/L magnesium chloride, 5 mmol/L EDTA, 2mmol/L PMSF, 10 µg/µL pepstatin and 10 µg/µL leupeptin and then lysed by 3 cycles of snap-freezing in liquid nitrogen and thawing. Values were corrected for protein concentration.

Apoptosis and necrosis determination by Acridine orange and Sytox green nuclear staining
Cells were seeded in 12-well plates and treated as indicated. Apoptosis was determined by assessment of nuclear condensation using Acridine orange staining (Sigma-Aldrich) at 2.5 µg/mL. Percentage of apoptotic cells was determined by counting the number of condensed nuclei and the total number of nuclei per field. Percentages are mean of two randomly chosen fields per condition.

To estimate necrosis, HSCs were incubated with Sytox green nucleic acid staining (Invitrogen) at 125 nmol/L in combination with Hoechst 33342 (Roche) at 5 μg/mL. Sytox green penetrates cells with leaky plasma membranes, a hallmark of necrotic cells, but does not cross the plasma membranes of viable or apoptotic cells. Hoechst 33342 crosses the plasma membrane of all cells. Percentage of necrotic cells was calculated by counting the total number of cells in two randomly chosen fields and counting the number of necrotic cells in the same fields.
Cells were monitored over time using an Olympus CKX41 microscope at 450-490 nm.

Caspase-3-like enzyme activity assay
Caspase-3-like enzyme activity was assayed as described before (4). Protein samples were obtained as described for the glutathione assay. Samples were corrected for protein concentration.

WST-1 Assay
Metabolic activity of HSCs was determined using WST-1 reagent (Roche). Cells were cultered in 24-well plates and treated as indicated. The protocol was performed according to the manufacturer's instructions. For measuring the optical density at 450 nm, 100 μL of conditioned medium per sample was transferred to a transparent 96-well plate.

Proliferation assay
Proliferation of HSCs was determined using the Cell Proliferation ELISA (Roche) kit, a chemiluminescent ELISA-based detection of BrdU incorporation, according to the manufacturer's instructions.

Western blot analysis
Western blot analysis was performed on HSCs harvested in SDS-PAGE sample buffer. The 2D-Quant protein assay (Amersham, Roosendaal, The Netherlands) was routinely performed to determine protein concentration for equal loading. Western blot analysis by SDS-PAGE was followed by transfer to Hybond ECL nitrocellulose membrane (Amersham) using a semi-dry blotting system. Ponceau S staining was used to check for equal electrophoretic transfer of proteins. After analysis of phosphorylated ERK1/2 using a monoclonal antibody (New England Biolabs, Leusden), phosphorylated p38 using a polyclonal antibody (Cell Signaling, Danvers, USA) and phosphorylated JNK using a monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, USA) blots were stripped for incubation with antibodies against total ERK1/2 using a polyclonal antibody (Santa Cruz Biotechnology), total p38 using a polyclonal antibody (Cell Signaling) or total JNK using a polyclonal antibody (Cell Signaling). After western blot analysis for PCNA using a monoclonal antibody (Novocastra, Newcastle upon Tyne, United Kingdom), blots were stripped and probed for β-actin using a monoclonal antibody (Sigma-Aldrich) to check equal loading. Secondary antibodies were either anti-mouse or anti-rabbit immunoglobulins conjugated with horseradish peroxidase. Immunoreactive bands were developed using enhanced chemiluminescence reagent (Cell Signaling) in combination with the quantity One system (Bio-rad). Blots were stripped using 0.1% SDS in PBS/0.1% Tween-20 at 65 °C for 30 minutes. Band intensities were quantified using the Quantity One software. Phosphorylated MAP kinase signals or PCNA were normalized against total MAP kinase signals or β-actin signal respectively. Data are expressed as relative optical density compared to control.

Statistical analysis
Statistical analysis of data was performed using SPSS 12.2. Data are presented as means ± standard deviation, unless otherwise indicated. Statistical differences between groups were

calculated using the non-parametric Kruskal-Wallis test, followed by a Mann-Whitney-U- test. P-value's below 0.05 were considered significant. Positive and negative controls were excluded in the statistical analyses.

Results

Reactive oxygen species induce heme oxygenase-1 expression in hepatic stellate cells
To investigate whether the reactive oxygen species (ROS) used in this study exert oxidative stress on HSCs, we determined the mRNA level of heme oxygenase-1 (HO-1), an oxidative stress responsive gene. Both menadione and hydrogen peroxide strongly induced HO-1 mRNA levels after 4 hours of stimulation (Figure 1A, B).

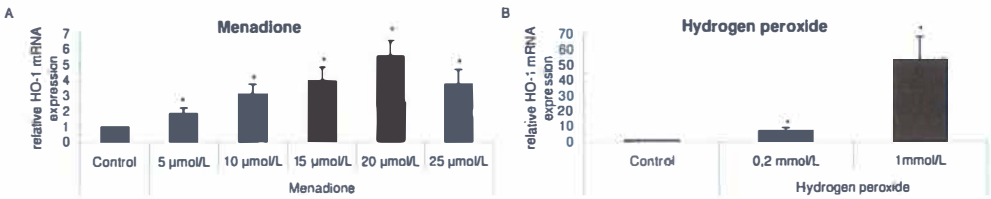

Figure 1. Reactive oxygen species induce the oxidative stress response gene HO-1. HSCs were treated with various concentrations of menadione or hydrogen peroxide for 4 hours. A) Representative experiment demonstrating dose-dependent induction of HO-1 mRNA levels by menadione. B) Representative experiment depicting the effect of hydrogen peroxide on HO-1 mRNA levels. Relative mRNA levels were normalized against 18S and untreated control levels were set to 1. * Significantly different from control, $p<0.05$. All experiments were performed four times, yielding identical results.

Menadione but not hydrogen peroxide reduces the glutathione pool in hepatic stellate cells
Another indicator of oxidative stress is the depletion of the cellular glutathione pool. Menadione induced a time- and dose-dependent decrease of both reduced and total glutathione in HSCs (Figure 2A,B). In contrast, 1 mmol/L hydrogen peroxide showed no effect on either total or reduced glutathione pools (Figure 2C).

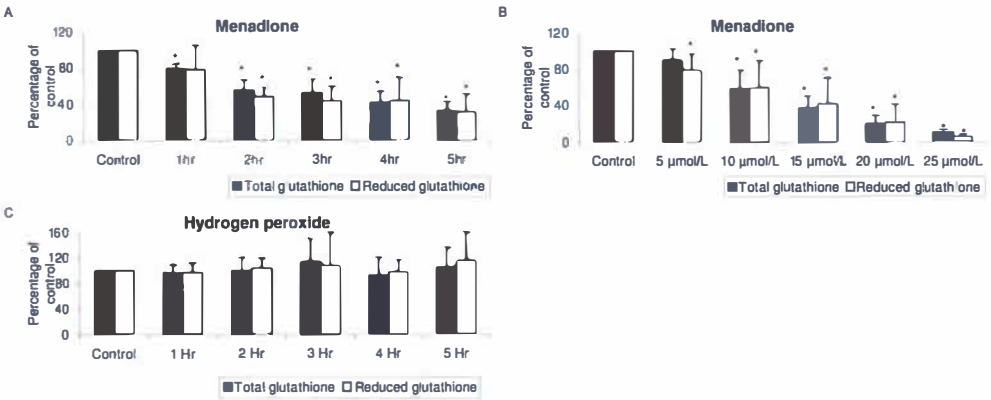

Figure 2. Menadione, but not hydrogen peroxide, reduces the glutathione pool of hepatic stellate cells. A) Time-dependent decrease of total and reduced glutathione pools after treatment with 20 µmol/L menadione B) Dose-dependent decrease of total and reduced glutathione pools after 5 hrs of treatment with menadione C) Hydrogen peroxide at 1 mmol/L had no effect on total and reduced glutathione pools. Controls were set to 100, results were shown as mean ± standard deviation of four independent experiments.* Significantly different from control, $p<0.05$.

Differential effects of menadione and hydrogen peroxide on hepatic stellate cell death

Menadione dose-dependently induces apoptosis of HSC (Figure 3A). The percentage of apoptotic cells after exposure to 15, 20, and 25 µmol/L menadione for 5.5 hours were 64%, 88%, and 99%, respectively. Menadione did not induce necrosis at any of the concentrations tested (data not shown). Pre-treatment of HSCs with PEG-SOD or the cell permeable glutathione donor GSH-MEE before exposure to 20 µmol/L menadione reduced menadione-induced apoptotic cell death from 88% to 21% and 9%, respectively (Figure 3B).

Hydrogen peroxide did not induce death of HSC at concentrations up to 1 mmol/L. Necrotic cell death of HSC only occurred after exposure of HSC to 5 mmol/L hydrogen peroxide. This toxicity of 5 mmol/L hydrogen peroxide was completely abolished by the addition of PEG-catalase (Figure 3C). Hydrogen peroxide did not induce apoptosis at any of the concentrations tested (data not shown).

Menadione-induced hepatic stellate cell apoptosis is caspase-independent

Inhibition of caspase 3, 6, or 9 did not inhibit menadione-induced apoptosis, as assessed by nuclear condensation (Figure 3D). In contrast, inhibition of caspase 3, 6, or 9 did inhibit menadione-induced apoptosis of rat hepatocytes (data not shown) and (4).

Furthermore, menadione did not induce caspase-3 activity in HSC (Figure 3E) after 5 hours exposure, in contrast to hepatocytes (after 8 hr exposure). This is in line with the lack of effect of caspase inhibitors on menadione-induced stellate cell apoptosis.

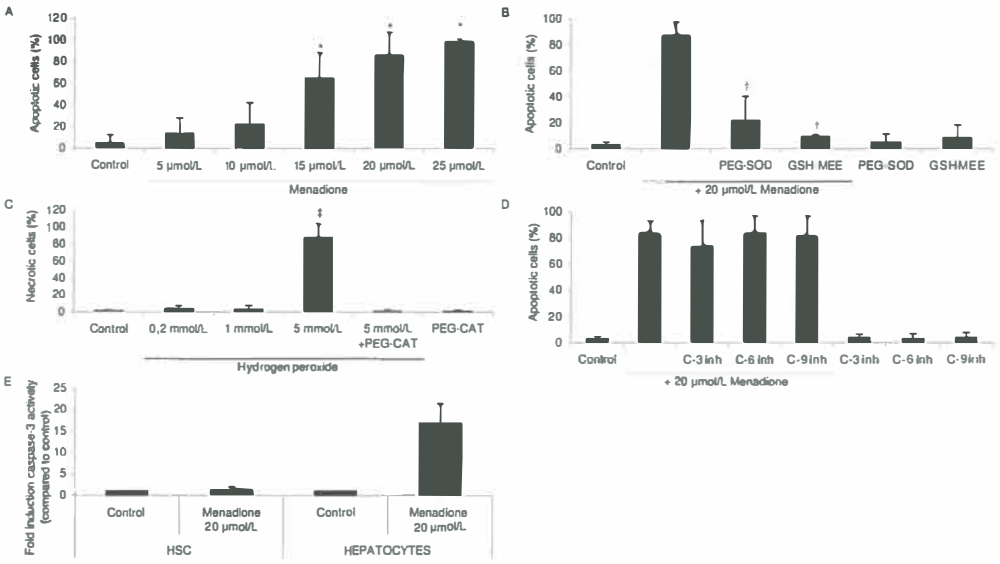

Figure 3. Menadione induces caspase-independent apoptosis, whereas hydrogen peroxide induces necrosis of HSCs. A) Menadione dose-dependently induced apoptosis of HSCs at 5.5 hrs. Apoptosis was determined by scoring condensed nuclei using acridine orange staining. B) PEG-SOD and the glutathione donor GSH-MEE significantly reduced apoptosis induced by 20 µmol/L menadione. C) Hydrogen peroxide induced necrotic cell death at 5 mmol/L. Necrosis was determined by scoring Sytox green positive nuclei and Hoechst 33442 positive nuclei. D) Menadione-induced apoptosis was caspase independent since the inhibitors for caspase 3, 6 or 9 did not prevent apoptosis induced by 20 µmol/L menadione. E) Menadione fails to induce caspase-3 activity in HSC after 5 hrs of exposure. * Significantly different from control, 5 µmol/L menadione and from 10 µmol/L menadione $p<0.05$. † Significantly different from 20 µmol/L menadione, $p< 0.05$, ‡ Significantly different from all other conditions, $p< 0.01$.

ROS-induced reduction of metabolic activity correlates with induction of cell death

Exposure to menadione resulted in a concentration-dependent decrease of metabolic activity, which correlated with the induction of apoptosis (Figure 4A). Hydrogen peroxide at 0.2 mmol/L had no effect on metabolic activity, while 1 mmol/L hydrogen peroxide slightly but significantly reduced metabolic activity. Cells exposed to 5 mmol/L hydrogen peroxide showed hardly any metabolic activity, reflecting the massive necrotic cell death at this concentration. PEG-catalase completely restored the metabolic activity of HSCs exposed to 5 mmol/L hydrogen peroxide (Figure 4B).

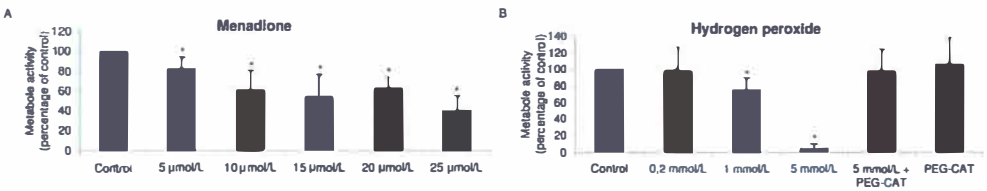

Figure 4. Reduction of metabolic activity correlates with induction of cell death. A) Menadione dose dependently decreased metabolic activity after 5 hr in HSCs. B) Metabolic activity was virtually absent in HSCs after 24 hrs treatment with 5 mmol/L hydrogen peroxide, whereas 0.2 mmol/L had no effect and 1 mmol/L showed a slight decrease in metabolic activity. Results were shown as mean ± standard deviation of at leased four independent experiments. * Significantly different from control, $p < 0.05$.

Reactive oxygen species inhibited serum- and PDGF-induced proliferation of hepatic stellate cells

Next, the effect of ROS on stellate cell proliferation induced by 20% serum or PDGF (in serum free conditions) was investigated. Non-apoptotic concentrations of menadione (5 and 10 µmol/L) had no effect on serum-induced proliferation of HSCs (Figure 5A). In contrast, hydrogen peroxide at 0.2 mmol/L had a slight but significant reducing effect on proliferation induced by 20% serum, whereas 1 mmol/L hydrogen peroxide completely blocked serum-induced proliferation (Figure 5B). As indicated earlier, the metabolic activity of cells exposed to 1 mmol/L hydrogen peroxide is still 75% of control cells (Figure 4B), indicating that these cells are still viable and metabolically active.

Slightly different effects of ROS on PDGF-induced proliferation were noted: menadione at 10 µmol/L partially inhibited PDGF-induced proliferation, whereas 5 µmol/L menadione had no effect (Figure 5C). Hydrogen peroxide at 1 mmol/L completely blocked PDGF-induced proliferation of HSC, whereas 0.2 mmol/L hydrogen peroxide had no effect (Figure 5D). Menadione alone (5 and 10 µmol/L) and hydrogen peroxide alone (0.2 and 1 mmol/L) did not significantly modulate HSC proliferation in serum free conditions.

The expression of the proliferating cell nuclear antigen (PCNA), correlated well with the results of the proliferation assays: the expression of PCNA was elevated in PDGF-stimulated HSCs and reduced when cells were co-treated with 1 mmol/L hydrogen peroxide, but not with 0.2 mmol/L hydrogen peroxide (Figure 5E).

Reactive oxygen species activate MAP kinases

Next, we investigated whether signaling pathways of the MAP kinase family are involved in ROS-induced effects on stellate cell death and proliferation. Menadione, at all concentrations tested, induced phosphorylation of p38 (Figure 6A). Menadione did not induce phosphorylation of ERK (Figure 6C). Only apoptotic concentrations of menadione (20 and 25 µmol/L) induced phosphorylation of JNK2 (Figure 6E). Hydrogen peroxide induced phosphorylation of p38, ERK and JNK2 (Figure 6B,D and F respectively).

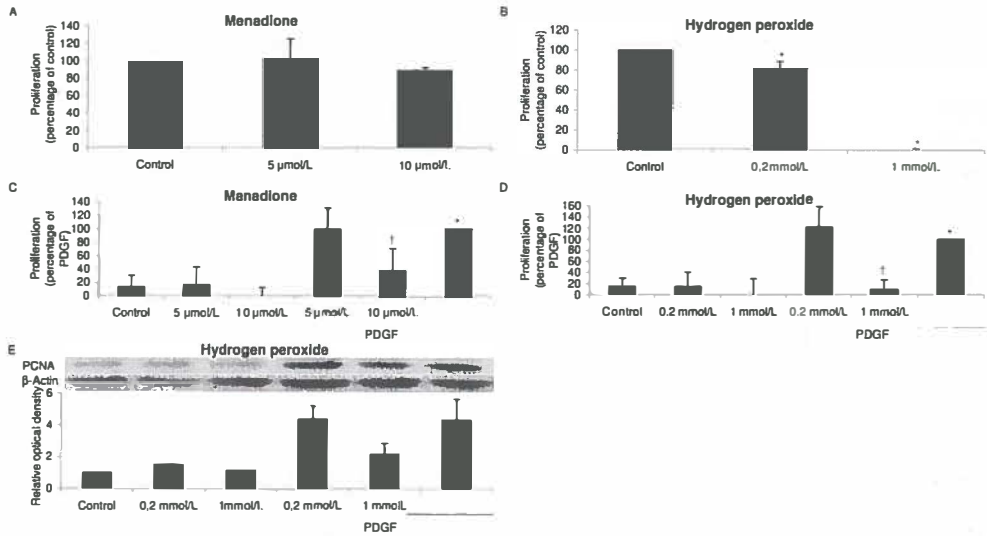

Figure 5. Reactive oxygen species inhibit serum- and PDGF-induced proliferation of hepatic stellate cells. A) Low, nontoxic concentrations of menadione had no effect on 20% serum-induced HSC proliferation. B) The nontoxic concentration of 1 mmol/L hydrogen peroxide completely blocked proliferation, whereas 0.2 mmol/L hydrogen peroxide had a slight inhibitory effect on serum-induced proliferation. C) At the nontoxic concentration of 10 µmol/L, menadione partially inhibited PDGF-induced proliferation of activated HSC, whereas 5 µmol/L menadione had no effect. D) 1 mmol/L hydrogen peroxide completely inhibited PDGF-induced proliferation, whereas 0.2 mmol/L had no effect. E) Expression of PCNA correlated with the proliferation assay. A representative experiment is depicted. Expression was normalized against β-actin expression. Results of the proliferation assay were shown as mean ± standard deviation of four independent experiments, either untreated controls or PDGF are set to 100.
* Significantly different from control, $p<0.05$. † Significantly different from PDGF, $p<0.05$.

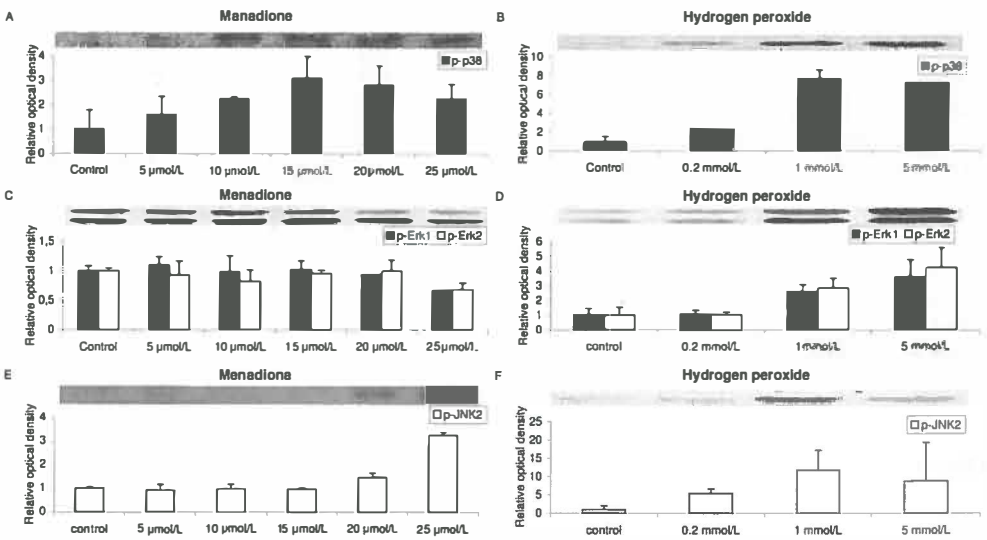

Figure 6. Reactive oxygen species induce phosphorylation of MAP kinases. Menadione phosphorylated p38 (A) and JNK (E), but had no effect on ERK phosphorylation (C). Hydrogen peroxide phosphorylated p38 (B), ERK (D) and JNK2 (F). Band intensities were quantified and phosphorylated MAP kinase signals were normalized against total MAP kinase signals. Data was expressed as fold-induction compared to control. Representative experiment of three independent experiments was depicted.

Effects of inhibition of MAP kinases on hepatic stellate cell death

Inhibition of the MAP kinase JNK using SP600125 partially (-35%) reduced menadione-induced apoptosis (Figure 7). Monitoring for 24 hours did not reveal a delay of apoptosis or a shift towards necrosis (data not shown). Inhibition of p38 using SB203580 or ERK using U0126 had no effect on menadione-induced cell death (Figure 7).

Inhibition of any of the MAP kinases JNK, p38 or ERK did not change the effect of 1 mmol/L or 5 mmol/L hydrogen peroxide on the viability of HSCs (data not shown).

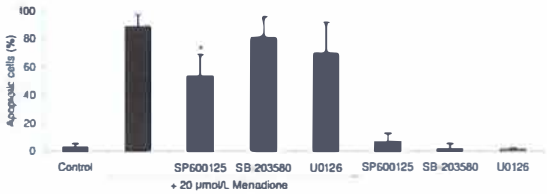

Figure 7. Activated JNK mediates menadione-induced apoptosis. Activated HSCs were pretreated for 30 minutes with the JNK-inhibitor SP600125, the p38 inhibitor SB-203580, or the ERK inhibitor U0126, followed by treatment with the toxic concentration of 20 µmol/L menadione for 5.5 hrs. SP600126 only partially inhibited apoptosis by approximately 35%. * Significantly different from 20 µmol/L menadione, $p < 0.05$.

Inhibition of MAP kinases modulates hepatic stellate cell proliferation

Inhibition of the various MAP kinases had different effects on serum-induced proliferation. Inhibition of p38 increased HSC proliferation, while inhibition of JNK had no effect on HSC proliferation. Inhibition of ERK decreased HSC proliferation (Figure 8A). Although inhibition of p38 stimulated HSC proliferation, it did not restore the decreased proliferation of HSCs treated with 1 mmol/L hydrogen peroxide. Likewise, inhibition of either JNK or ERK also did not restore HSC proliferation after treatment with 1 mmol/L hydrogen peroxide (Figure 8B).

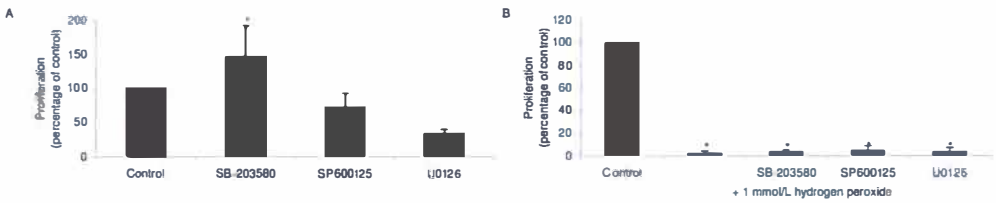

Figure 8. ERK and p38 have opposite effects on serum-induced proliferation of hepatic stellate cells. A) Serum-induced proliferation of activated HSCs was further increased after inhibition of p38 with SB-203580. Proliferation rate was not significantly changed after treatment with the JNK-inhibitor SP600125. The ERK-inhibitor U0126 decreased proliferation of activated HSCs. B) The reduction of serum-induced HSC proliferation by 1 mmol/L hydrogen peroxide was not restored by any of the MAP kinase inhibitors. Untreated controls were set to 100%, results were shown as mean ± standard deviation of four independent experiments. * Significantly different from control, $p<0.05$.

Discussion

Oxidative stress has been implicated in the activation and proliferation of hepatic stellate cells and hence, the development of liver fibrosis. However, there are conflicting data in the literature regarding the effects of reactive oxygen species on stellate cell proliferation and viability. Therefore, this study was designed to analyze in detail the effects of two reactive oxygen species on stellate cell proliferation and viability. In our study, we used concentrations of hydrogen

peroxide and menadione that are commonly used in the literature (5, 15, 16).
The most important findings of our study are that reactive oxygen species do not directly induce stellate cell proliferation, but rather block stellate cell proliferation and induce cell death. Other important findings of our study are that superoxide anion (menadione) induced stellate cell apoptosis is caspase-independent and can be prevented by glutathione.
Both superoxide anions and hydrogen peroxide induced oxidative stress in HSCs as indicated by the induction of the oxidative stress responsive gene heme oxygenase-1 (HO-1). The reduced induction of HO-1 using 25 μmol/L menadione is most likely due to massive cell death observed at this concentration.
Glutathione depletion is only observed after exposure to menadione, but not after exposure to hydrogen peroxide. Menadione is known to interact with reduced glutathione forming a menadione-GSH conjugate (20). This conjugate can be formed in vitro and will still be able to produce superoxide anions, while at the same time the conjugation changes it biological properties in being water-soluble and easier removable out of the cells (21). Conjugation of hydrogen peroxide to GSH is not known, and not expected since it is already water-soluble.
Replenishing glutathione protected the cells from menadione-induced apoptosis, without a shift toward necrosis or delayed apoptosis, implying true protection. This has also been observed for an hepatic stellate cell line (22) and in another cell type (Jurkat cell line) using FasLigand as an inducer of cell death (23). This is the first time that glutathione-dependent protection against cell death is described in primary HSCs.
Recently, it has been reported that loss of glutathione, via the ABC-transporter Mrp1, is a necessary signal for apoptosis (24). Therefore, we hypothesize that menadione induced loss of glutathione, either via oxidation of glutathione or via conjugation of glutathione to menadione (20), might signal apoptotic cell death. This is supported by our finding that the glutathione-donor, GSH-MEE, prevents menadione-induced apoptosis. Furthermore, we recently described the expression of the ABC-transporter Mrp1 in hepatic stellate cells (25). Since hydrogen peroxide does not deplete cellular glutathione and does not induce apoptotic cell death, our results suggest that the cellular glutathione status is an important determinant in the regulation of apoptosis in hepatic stellate cells.
Another important finding of this study is that superoxide anion-induced apoptosis of hepatic stellate cells is caspase-independent: menadione did not induce caspase-3 activity and caspase inhibitors did not inhibit apoptosis. Previously, we showed that menadione-induced apoptosis in hepatocytes is caspase dependent (4) and this was confirmed in the present study. Our results are in contrast to a previous study, demonstrating caspase-dependency of superoxide anion-induced stellate cell apoptosis (14). In this study, the caspase-3 inhibitor DEVD-fmk and the caspase-9 inhibitor LHED-fmk only partially reduced apoptosis by approximately 33% and 15% respectively. Furthermore, in the same study, the authors report that neither caspase-8 nor caspase-9 could be detected in stellate cells and they used very high concentrations of inhibitors (20 μmol/L), that are likely to inhibit non-caspase proteases as well. We used 100-400x lower concentrations, that were still able to inhibit bile-acid and menadione-induced apoptosis in hepatocytes.
Our results indicate that the response to superoxide anions is cell type-specific. This knowledge could be used to achieve cell-type selective effects when contemplating the use of caspase-inhibitors in chronic liver diseases: caspase inhibitors will protect hepatocytes against oxidative stress, but not hepatic stellate cells.
In our in vitro study, both hydrogen peroxide and superoxide anions (menadione) did not induce HSC proliferation. In fact, hydrogen peroxide inhibited both serum and PDGF-induced stellate cell proliferation, whereas menadione inhibited only PDGF-induced, but not serum-induced

stellate cell proliferation. This latter discrepancy could be due to the fact that serum contains additional growth factors (other than PDGF) that may induce HSC proliferation and that act via different receptors, e.g. EGF and IGF-1. Alternatively, the concentration of PDGF in serum might have been higher than the concentration used for PDGF alone.

The effects of ROS on HSC proliferation have been extensively studied and have yielded contradictory results. These conflicting results may be due to the use of a wide variety of ROS generating systems. Lipid peroxidation products such as isoprostanes have been proposed to stimulate stellate cell proliferation directly (26). However, conflicting data exists in this regard as well: some have argued that lipid peroxidation products induce apoptosis of stellate cells (27) whereas others have shown no effect on proliferation (28). We show in our study that the intracellular generation of superoxide anions or the acute exposure to high (extracellular) concentrations of hydrogen peroxide do not stimulate stellate cell proliferation. Toxic effects appear only at very high concentrations. These findings indicate that stellate cells are unusually resistant to ROS-induced toxicity and that oxidative stress-associated proliferation of stellate cells is due to other factors (aldehydes, alkenals, growth factor-induced ROS-production, NADPH-oxidases) or dependent on the kinetics and location of ROS generation.

Important regulators of cell survival and cell proliferation are members of the MAP kinase family (p38, JNK, ERK). An interesting observation of our study is the differential activation of MAP kinases by hydrogen peroxide and menadione. Both hydrogen peroxide and menadione activate JNK and p38 and hydrogen peroxide also activates ERK (Fig. 6D). Hydrogen peroxide-induced phosphorylation of JNK, p-38 and ERK has also been described by Kikuta et al. (29) in pancreatic stellate cells.

We also demonstrate that menadione-induced JNK activation is at least partially responsible for menadione-induced apoptosis of stellate cells. Kamata et al. (30) also showed that ROS activate JNK and that sustained JNK activation is necessary for the induction of apoptosis in mouse fibroblasts. In contrast, we observed that inhibition of hydrogen peroxide-induced JNK activation does not prevent necrotic cell death.

With respect to the involvement of MAP kinases in stellate cell proliferation, our results are also intriguing. Inhibition of p38, in the absence of ROS, increases HSC proliferation. This is in line with the results of Schnabl et al. (31). A possible explanation of this effect is the inhibitory effect of p38 on cyclin DI in the regulation of the cell cycle as described by Lavoie et al. (32) in lung fibroblasts. Schnabl et al. (31) also showed decreased proliferation after JNK inhibition using a dominant negative adenoviral construct. This construct acts upstream of JNK on TGF-β-activated kinase 1 (TAK1). Schnabl et al. (31) observed a similar effect using the inhibitor SP600125. We could not confirm this finding with the JNK inhibitor SP600125, although we observed a similar trend that did not reach statistical significance. ERK inhibition clearly decreased HSC proliferation. These data suggest that activation of ERK promotes proliferation of hepatic stellate cells, whereas activation of p38 inhibits proliferation.

In this respect, the data of Li et al. are very interesting (33). Li et al. demonstrated that activated p38 regulates HO-1 induction (34) and that HO-I induction is anti-proliferative. Our study supports and extends these data: 1) Both menadione and hydrogen peroxide activate p38 and this was shown to be anti-proliferative (Figure 8A). 2) We demonstrate a correlation between p38 activation (Figure 6A,B) and HO-1 induction (Figure 1 A,B). We also show that ERK-phosphorylation is induced by hydrogen peroxide (Figure 6F). Although ERK phosphorylation promotes proliferation (Figure 8A), this proliferation-stimulating effect is most likely overruled by the activation of p38 and its subsequent anti-proliferative effect via induction of HO-I. Our data correlate very well with the study by Novo et al. (15) showing that superoxide anions, generated by hypoxantine/xanthine oxidase, induce apoptosis, whereas hydrogen peroxide

induces necrosis of activated human HSCs at very high concentrations. Thirunavukkarasu et al. (14) also demonstrated that superoxide anions generated by hypoxantine/xanthine oxidase induce apoptosis in activated rat HSCs. Our observation that reactive oxygen species inhibit serum- or PDGF-induced proliferation of HSCs extends the observation of Novo et al. (15).
Our results clearly demonstrate that hydrogen peroxide and menadione do not directly induce stellate cell proliferation. Therefore, in chronic liver injury accompanied by oxidative stress, stellate cell activation and proliferation is most likely induced by other factors, e.g.like lipid peroxidation products (10, 35) and inflammatory cytokines or growth factors released by inflammatory cells, including Kupffer cells (36). In addition, the source and site of production of ROS may influence the effects of ROS on cell viability and proliferation. In this respect it is important to note that the growth factor-induced activation of the plasma membrane-bound ROS-generating enzyme NADPH-oxidase stimulates HSC proliferation (37-39). The results of our study indicate that the association between oxidative stress and fibrosis cannot be due to direct effects of ROS on activated hepatic stellate cells. Whether ROS generation during liver fibrogenesis actually slows down stellate cell activation and fibrogenesis is speculative and remains to be determined. Indirect effects of ROS generation during fibrogenesis, e.g. inflammation, may actually promote HSC proliferation.

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Chapter 4

Glutathione and antioxidant enzymes serve complementary roles in protecting activated hepatic stellate cells against hydrogen peroxide-induced cell death

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Submitted

Abstract

Background
In chronic liver disease, hepatic stellate cells (HSCs) are activated, proliferate, and are exposed to elevated levels of reactive oxygen species (ROS).

Aim
To investigate the protective mechanisms of activated HSCs against ROS-induced toxicity.

Methods
Culture-activated rat HSCs were exposed to hydrogen peroxide. Apoptosis and necrosis were determined by acridine orange and Sytox Green nuclear staining, respectively. The hydrogen peroxide detoxifying enzymes catalase and glutathione-peroxidase (GPx) were inhibited using 3-amino-1,2,4-triazole and mercaptosuccinic acid, respectively. The anti-oxidant glutathione was depleted by using L-buthionine-sulfoximine and repleted with the GSH-analogue GSH-monoethylester (GSH-MEE).

Results
Upon activation, HSCs increase their cellular glutathione content and GPx expression. Hydrogen peroxide (0.2-1 mmol/L) did not induce cell death. Glutathione depletion dramatically increased sensitivity of HSCs to hydrogen peroxide, resulting in 35% and 75% necrotic cells at 0.2 and 1 mmol/L hydrogen peroxide, respectively. This sensitizing effect was abolished by GSH-MEE. Inhibition of catalase or GPx significantly increased hydrogen peroxide-induced apoptosis but not necrosis. This apoptosis was not reversed by GSH-MEE.

Conclusion
Activated HSCs have increased ROS-detoxifying potential compared to quiescent HSCs. Glutathione is an important determinant in the protection against ROS-induced HSC necrosis, whereas hydrogen peroxide detoxifying enzymes protect against apoptotic cell death.

Introduction

Oxidative stress is defined as the imbalance between pro-oxidants and antioxidants. Under normal conditions, reactive oxygen species are detoxified by various enzymatic and non-enzymatic antioxidants. When pro-oxidants exceed the antioxidant capacity of the cell, oxidative stress is the result. Prolonged oxidative stress in the liver is associated with liver fibrosis and cirrhosis. Liver fibrosis is characterized by the loss of hepatocytes and the activation of hepatic stellate cells (HSCs). During the activation process quiescent HSCs transform into proliferating myofibroblast-like cells. Unlike quiescent HSCs, these activated cells lack retinoid-storing capacity, produce excessive amounts of connective tissue and proliferate(1).
Although generation of reactive oxygen species has been associated with the activation of stellate cells and liver fibrosis, hardly anything is known about the role of various antioxidant systems in activated HSCs. Hydrogen peroxide is generated in the mitochondria by several enzymes, like NADPH-oxidases and xanthine oxidase, and also in the detoxification of superoxide anions by superoxide dismutases like the cytosolic CuZn-SOD (SOD1) and the mitochondrial Mn-SOD (SOD2) (2, 3). In addition, hydrogen peroxide can be generated extracellularly by inflammatory cells, e.g. neutrophils (2). Hydrogen peroxide is detoxified by the peroxisomal catalase or the cytosolic glutathione peroxidase. Glutathione peroxidase converts reduced glutathione (GSH) into oxidized glutathione (GSSG) (2). To control the hydrogen peroxide level within the cell, the cell has to balance the activity of catalase and glutathione peroxidases relative to SODs. The aim of this study was to investigate the role of antioxidant systems in the protection against hydrogen peroxide-induced toxicity in activated stellate cells.

Materials and methods

Animals
Specified pathogen-free male Wistar rats were purchased from Harlan (Zeist, the Netherlands). They were housed under standard laboratory conditions and had free access to standard laboratory chow and water. Each experiment was performed following the guidelines of the local committee for care and use of laboratory animals.

Hepatic stellate cell isolation and culture
Hepatic stellate cells (HSCs) were isolated from male Wistar rats (500-600 g) by pronase (Merck, Amsterdam, the Netherlands) and collagenase-P (Roche, Almere, the Netherlands) perfusion of the liver, followed by Nycodenz (Axis-Shield POC, Oslo, Norway) gradient (12% w/v) centrifugation as described previously(4). Cells were then cultured in Iscove's Modified Dulbecco's Medium with Glutamax (Invitrogen, Breda, the Netherlands) supplemented with 20% heat-inactivated fetal calf serum (Invitrogen), 1 mmol/L sodium pyruvate (Invitrogen), 1x MEM non essential amino acids (Invitrogen), 50 µg/mL gentamycin (Invitrogen), 100 U/mL penicillin (Lonza, Vervier, Belgium), 10 µg/mL streptomycin (Lonza), 250 ng/mL fungizone (Lonza) and 250 U/mL Nystatin (Sanofi-Synthelabo, Maassluis, the Netherlands) in a humidified atmosphere containing 5% CO2 at 37 °C. For studying activation of hepatic stellate cells, cells were seeded, grown to confluency and harvested at the indicated time points. Primary HSC cultures were passaged via trypsinization, and then cultured in Iscove's medium with supplements as described above, except Nystatin. Prior to experiments, HSCs were serum-starved for 24 hours, unless indicated otherwise.

Experimental design
HSCs were activated by culture for at least 14 days. These activated rat HSCs were exposed to oxidative stress induced by 0.2 or 1 mmol/L hydrogen peroxide (Merck). The glutathione depleting compound L-buthionine-sulfoximine (BSO, Sigma) was used at 200 µmol/L. The cell permeable glutathione donor GSH-monoethylester (GSH-MEE, Calbiochem) was used at 5 mmol/L. The glutathione peroxidase inhibitor mercaptosuccinic acid (MS, Sigma-Aldrich) was used at 10 mmol/L and the catalase inhibitor 3-amino-1,2,4-triazole (3AT, Sigma-Aldrich) was used at 20 mmol/L. The caspase-3 inhibitor (Z-DEVD-FMK, R & D Systems) was used at 0.05 µmol/L. Inhibitors were added 30 minutes prior to exposure to hydrogen peroxide, with the exception of BSO, which was added 17-20 hours prior to exposure to hydrogen peroxide.

Glutathione assay
Glutathione and glutathione disulfide content was determined using a spectrophotometry-based assay as described previously (5). HSCs were harvested in a lysis buffer composed of 25 mmol/L HEPES, 5 mmol/L MgCl2, 5 mmol/L EDTA, 2 mmol/L PMSF, 10 µg/µL pepstatin and 10 µg/µL leupeptin and then lysed by 3 cycles of snap-freezing in liquid nitrogen and thawing. Values were corrected for protein concentration, determined by the BioRad DC protein assay (Veenendaal, the Netherlands) according to the manufacturer's instructions.

RNA isolation
RNA was isolated using Tri-reagent (Sigma-Aldrich) according to the manufacturer's instructions. Reverse transcription was performed on total RNA using random nonamers (Sigma-Aldrich) in a final volume of 50 µl. Reverse transcription was performed in three steps: 10 minutes at 25°C, 1 hour at 37°C and 5 minutes at 95°C.

Quantitative Real-Time PCR
Real time detection was performed on the ABI PRISM 7700 (PE Applied Biosystems) initialized by 10 min at 95°C, followed by 40 cycles (15 seconds at 95°C, and 1 minute at 60°C). Each sample was analyzed in duplicate. mRNA levels of 18S were used as an internal control. Reaction mixture contained qPCR mastermix plus-dTTP (Eurogentec, Maastricht, the Netherlands) supplemented with 900 nmol/L sense and anti-sense primer and 200 nmol/L labeled probe. The primers (Invitrogen) and probe (Eurogentec) used are listed in Table 1. Relative gene expressions were calculated using the ΔΔCt method.

Apoptosis and necrosis determination by Acridine orange and Sytox green/Hoechst 33342 nuclear staining
Cells were seeded in 12-well plates and treated as indicated. Apoptosis was determined by assessment of nuclear condensation using Acridine orange staining (Sigma-Aldrich) at 2.5 µg/mL. After 6 hours, the percentage of apoptotic cells was determined by dividing the number of condensed nuclei by total number of nuclei per field, amplified with 100. Percentages are the mean of two randomly chosen fields per condition.
To determine necrosis, HSCs were incubated with Sytox green nucleic acid staining (Invitrogen) at 125 nmol/L in combination with Hoechst 33342 (Roche) at 5 µg/mL. Sytox green penetrates cells with leaky plasma membranes, a hallmark of necrotic cells, but does not cross the plasma membranes of viable or apoptotic cells. Hoechst 33342 crosses the plasma membrane of all cells.
After 3 hours the percentage of necrotic cells was determined by dividing the number of Sytox green positive nuclei by the number of Hoechst 33342 positive nuclei of the same field, amplified

with 100. Two randomly chosen fields were used to determine the average per condition. Cells were monitored using an Olympus CKX41 microscope at 450-490 nm.

Table 1. Sequences of rat PCR primers and probes used for real-time detection PCR analysis

Gene		Primers
18S	sense	5'-CGG CTA CCA CAT CCA AGG A-3'
	antisense	5'-CCA ATT ACA GGG CCT CGA AA-3'
	probe	5'-CGC GCA AAT TAC CCA CTC CCG A-3'
α-SMA	sense	5'-GCC AGT CGC CAT CAG GAA C-3'
	antisense	5'-CAC ACC AGA GCT GTG CTG TCT T-3'
	probe	5'-CTT CAC ACA TAG CTG GAG CAG CTT CTC GA-3'
Catalase	sense	5'-GGA TTA TGG CCT CCG AGA TCT-3'
	antisense	5'-ACC TTG GTC AGG TCA AAT GGA T-3'
	probe	5'-ATG CCA TCG CCA GTG GCA ATT ACC-3'
Collagen type 1	sense	5'-TGG TGA ACG TGG TGT ACA AGG T-3'
	antisense	5'-CAG TAT CAC CCT TGG CAC CAT-3'
	probe	5'-TCC TGC TGG TCC CCG AGG AAA CA-3'
GCL	sense	5'-GCC CAA TTG TTA TGG CTT TGA GT-3'
	antisense	5'-CCT CCC GTG TTC TAT CAT CTA CAG A-3'
	probe	5'-ACT CCC CAG CGA CAA TCA ATG TCT GAC AC-3'
Gpx1	sense	5'-GGA CAT CAG GAG AAT GGC AAG A-3'
	antisense	5'-CGC ACT TCT CAA ACA ATG TAA AGT TG-3'
	probe	5'-TTC CCT CAA GTA TGT CCG ACC CGG TG-3'
HO-1	sense	5'-CAC AGG GTG ACA GAA GAG GCT AA-3'
	antisense	5'-CTG GTC TTT GTG TTC CTC TGT CAG-3'
	probe	5'-CAG CTC CTC AAA CAG CTC AAT GTT GAG C-3'
CuZn SOD	sense	5'-CAG GAC CTC ATT TTA ATC CTC ACT C-3'
	anisense	5'-GTC TCC AAC ATG CCT CTC TTC A-3'
	probe	5'-CCG CTG GAC CGC CAT GTT TCT T-3'
MnSOD	sense	5'-CAC CGA GGA GAA GTA CCA CGA-3'
	antisense	5'-GAA CTT CAG TGC AGG CTG AAG A-3'
	probe	5'-CCT GAG TTG TAA CAT CTC CCT TGG CCA G-3'
TGF-β	sense	5'-GGG CTA CCA TGC CAA CTT CTG-3'
	antisense	5'-GAG GGC AAG GAC CTT GCT GTA-3'
	probe	5'-CCT GCC CCT ACA TTT GGA GCC TGG A-3'

Proliferation assay

Proliferation of HSCs was determined using the Cell Proliferation ELISA kit (Roche), a chemiluminescent ELISA-based detection of BrdU incorporation, according to the manufacturer's instructions.

Statistical analysis

Statistical analyses of data were performed using SPSS 14. Data are presented as mean ± standard deviation, unless otherwise indicated. Statistical differences between groups were calculated using the non-parametric Kruskal-Wallis test, followed by a Mann-Whitney-U-test. P-values below 0.05 were considered significant.

Results

Glutathione peroxidase 1 expression is induced during activation of hepatic stellate cells

Upon the transformation of quiescent stellate cells into activated stellate cells, marked changes in gene expression occur. Therefore, we first investigated the expression of various genes involved in the detoxification of reactive oxygen species during the activation process. mRNA expression of catalase was significantly induced after two days of culture, but decreased upon complete activation of stellate cells (Figure 1A). Manganese superoxide dismutase (Mn-SOD) expression was strongly reduced upon activation (Figure 1B), while the copper-zinc superoxide dismutase (CuZn-SOD) expression was unaltered (Figure 1C). Although a trend towards reduced heme oxygenase-1 (HO-1) expression was observed, this did not reach statistical significance (Figure 1D). Interestingly, mRNA expression of glutathione peroxidase 1 (GPx1) increases during activation of stellate cells (Figure 1E), while glutamate-cysteine ligase (GCL, a.k.a. γ-glutamylcysteine synthetase), the rate limiting enzyme in glutathione synthesis, was not altered upon stellate cell activation (Figure 1F). In accordance with literature, alpha-smooth muscle actin (α-SMA), a marker for stellate cell activation, was strongly induced upon activation (Figure 1G).

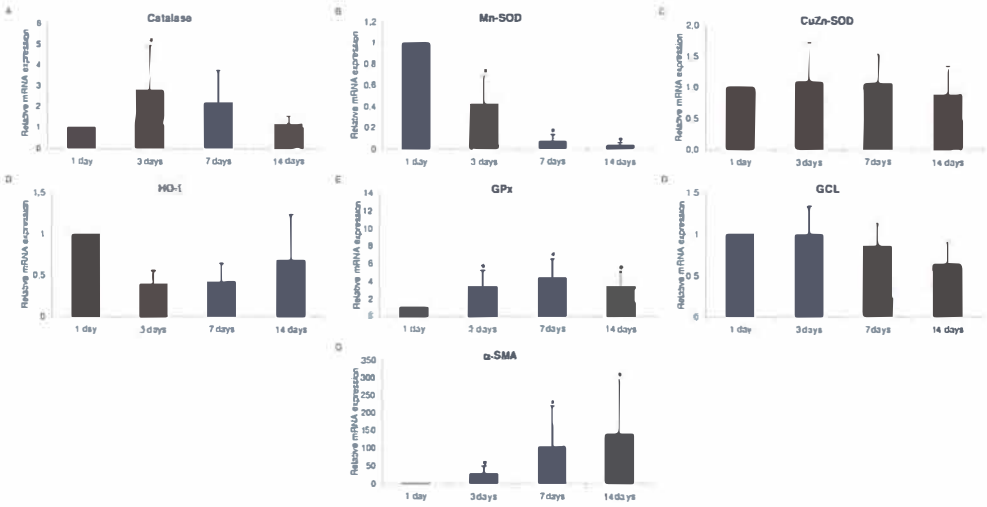

Figure 1. Glutathione peroxidase-1 is induced during hepatic stellate cell activation. Upon activation of hepatic stellate cells A) the expression of catalase was significantly induced after three days of culture, but decreases upon complete activation, B) the expression of Mn-SOD was strongly reduced, C) the expression of Cu/Zn-SOD did not changed, D) the expression of HO-1 was not significantly regulated, E) the expression of GPx1 increased, F) the expression of GCL did not changed, G) the expression of the activation marker α-SMA was strongly induced. *Significant difference compared to qHSC at 1 day, $p<0.05$

Activated hepatic stellate cells have a higher glutathione content than quiescent hepatic stellate cells

Total glutathione levels were determined in quiescent (day 1 after isolation) HSCs and in activated HSCs. The total cellular glutathione content was increased 5.6 times upon activation of hepatic stellate cells (Figure 2A), despite unchanged expression of GCL, the rate-limiting enzyme in the synthesis of glutathione (Figure 1F).

Depletion of glutathione in activated hepatic stellate cells does not induce cell death

Pre-incubation of activated, serum-starved HSCs with BSO reduced total glutathione levels by 87% (Figure 2B). Upon depletion of glutathione, no change in stellate cell morphology was observed and there was no increase in either necrotic or apoptotic cell death (data not shown). Likewise, BSO treatment also reduced glutathione content by 88% in activated HSCs cultured in medium containing 20% FCS (Figure 2C), without visible morphological changes.

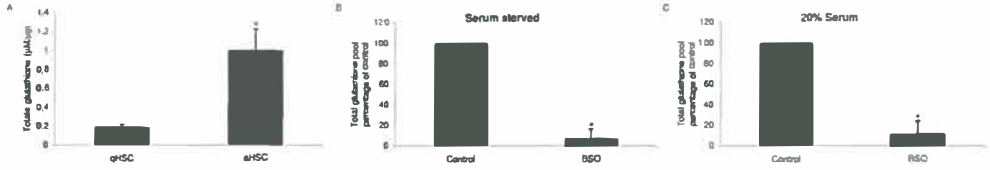

Figure 2. Total glutathione content is increased upon HSC activation. A) Activated HSCs (aHSC) have approximately 5.6 times higher glutathione levels than quiescent HSCs (qHSC). Results are shown as mean ± S.E.M of four independent experiments, *p<0.05 B) Pre-treating HSCs with BSO depleted cellular glutathione content by 87% in serum-starved HSCs. Results are shown as mean ± stdev of four independent experiments, *p<0.05. C) BSO treatment depleted glutathione levels by 88% in HSCs cultured in medium containing 20% FCS. Results are shown as mean ± stdev of four independent experiments, *p<0.05.

Glutathione depletion augments the induction of heme-oxygenase-1 and glutamate cysteine ligase mRNA in stellate cells

To investigate whether the depletion of glutathione leads to increased oxidative stress in stellate cells, we determined the mRNA level of the oxidative stress-responsive gene heme-oxygenase-1 (HO-1). Depletion of glutathione increased HO-1 mRNA levels 2.3 times. In addition, mRNA expression of GCL, the rate limiting enzyme in glutathione synthesis, was increased 1.6-fold. In contrast, glutathione depletion had no effect on the expression of the hydrogen peroxide-detoxifying enzymes GPx1 (Figure 3A) and catalase and the superoxide dismutases Mn-SOD, and CuZn-SOD (Figure 3B). Furthermore, glutathione depletion did not change the expression of the known markers of stellate cell activation α-smooth muscle actin (α-SMA), collagen type 1, and TGF-β (Figure 3C).

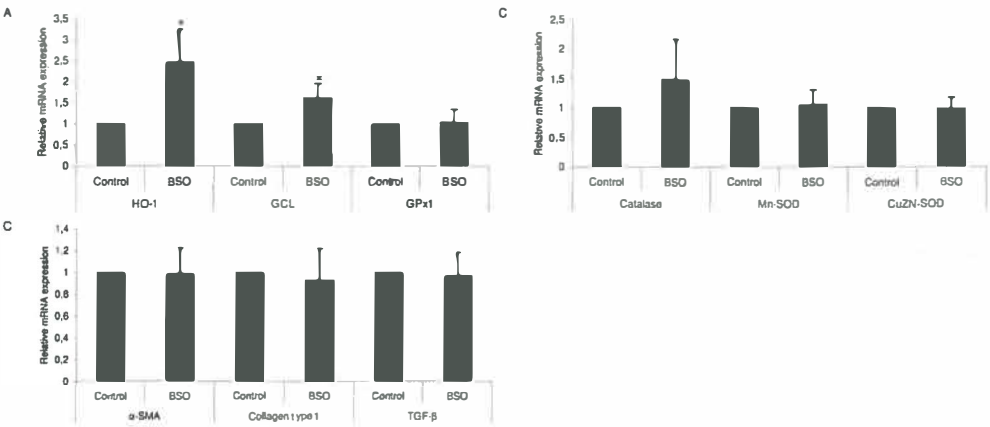

Figure 3. Glutathione depletion increases oxidative stress in activated hepatic stellate cells. A) Depleting glutathione with BSO induced HO-1 and GCL. However, glutathione depletion had no effect on (A) GPx1, (B) antioxidant enzyme expression of catalase, Mn-SOD, and CuZn-SOD, (C) the expression of HSC activation markers, α-SMA, collagen type 1 and TGF-β. Results are shown as mean ± stdev of at least four independent experiments, *p<0.05

Glutathione depletion does not alter hepatic stellate cell proliferation
Glutathione depletion did not affect hepatic stellate cell proliferation (Figure 4A). Moreover, supplementing glutathione with GSH-MEE also did not affect proliferation (data not shown).

Depletion of glutathione increases sensitivity to hydrogen peroxide induced necrosis
After depletion of glutathione with BSO, 35% and 75% of the cells became necrotic after exposure to, respectively, 0.2 and 1 mmol/L hydrogen peroxide for three hours. This indicates that BSO greatly enhanced sensitivity to hydrogen peroxide-induced necrosis. Restoration of glutathione content using GSH-MEE almost completely reversed hydrogen peroxide-induced necrosis in BSO-treated HSCs (Figure 4B,C).

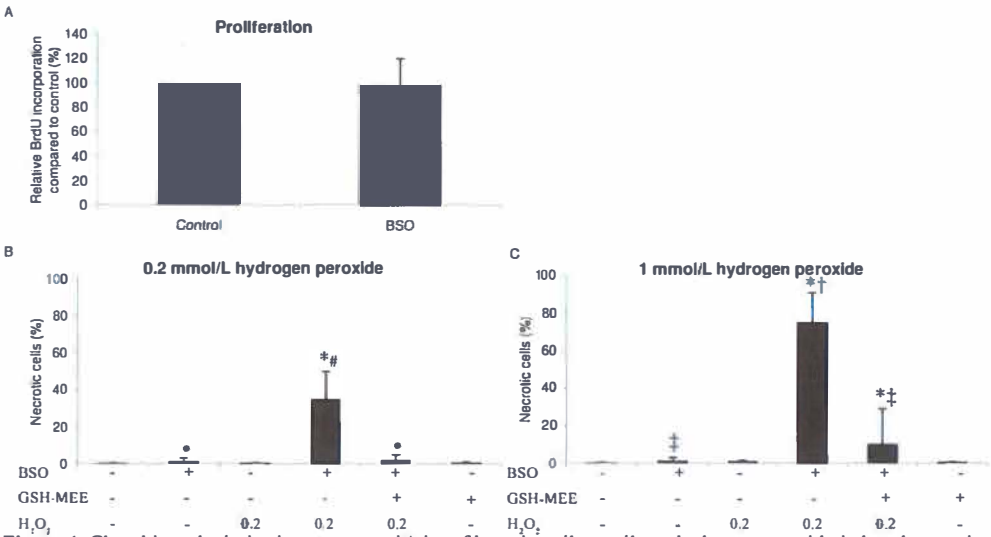

Figure 4. Glutathione depletion increases sensitivity of hepatic stellate cells to hydrogen peroxide-induced necrosis. A) Glutathione depletion with BSO had no effect on HSC proliferation. B) Glutathione depletion and subsequent exposure to 0.2 mmol/L hydrogen peroxide (H_2O_2) induced necrosis in 35% of the cells. C) Glutathione depletion and subsequent exposure to 1 mmol/L hydrogen peroxide induces necrosis in 75% of the cells. Results are shown as mean ± stdev of four independent experiments. *Significant difference compared to control, $p<0.05$; # Significant difference compared to 0.2 mmol/L hydrogen peroxide, $p<0.05$; •Significant difference compared to 0.2 mmol/L hydrogen peroxide + BSO, $p<0.05$; †Significant difference compared to 1 mmol/L hydrogen peroxide, $p<0.05$; ‡Significant difference compared to 1 mmol/L hydrogen peroxide + BSO, $p<0.05$.

Glutathione peroxidase protects against oxidative stress-induced apoptosis
The importance of the antioxidant enzyme GPx in the protection of activated HSCs against oxidative stress was investigated using the GPx inhibitor mercaptosuccinic acid (MS). This inhibitor significantly raised apoptosis (9%) in HSCs after 6 hours, even in the absence of exogenous hydrogen peroxide. Cells treated with 0.2 or 1 mmol/L hydrogen peroxide alone or 0.2 mmol/L hydrogen peroxide + MS, failed to significantly induce apoptosis. Combined treatment of MS and 1 mmol/L hydrogen peroxide induced significantly higher levels (28%) of apoptosis, compared to MS alone (Figure 5A). No effects on necrosis were measured during this experiment.

Catalase protects against oxidative stress-induced apoptosis
The importance of the antioxidant enzyme catalase in the protection of activated HSCs against oxidative stress was investigated using the catalase inhibitor 3-amino-1,2,4-triazole (3-AT).

This inhibitor significantly induced apoptotic cell death (18%) in HSCs after 6 hours, even in the absence of exogenous hydrogen peroxide. Cells treated with 0.2 or 1 mmol/L hydrogen peroxide in the presence of the inhibitor catalase showed even higher levels of apoptosis, 22% an 44% respectively (Figure 5B). No effects on necrosis were measured.

Inhibiting both glutathione peroxidase and catalase elevates apoptotic levels even further
Inhibiting both GPx and Catalase induced 22% apoptotic cells even in the absence of exogenous hydrogen peroxide. Exposing the cells to elevated levels of hydrogen peroxide induced apoptotic levels even further, 36% in co-treatment with 0.2 mmol/L hydrogen peroxide and 57 % in co-treatment with 1 mmol/L hydrogen peroxide (Figure 5C).
Increasing the glutathione content using the glutathione donor GSH-MEE did not influence this cell death (Figure 5D). Inhibition of caspase-3 decreased partially apoptosis of HSCs (Figure 5E). In none of the described conditions, necrotic cell death was observed (data not shown).

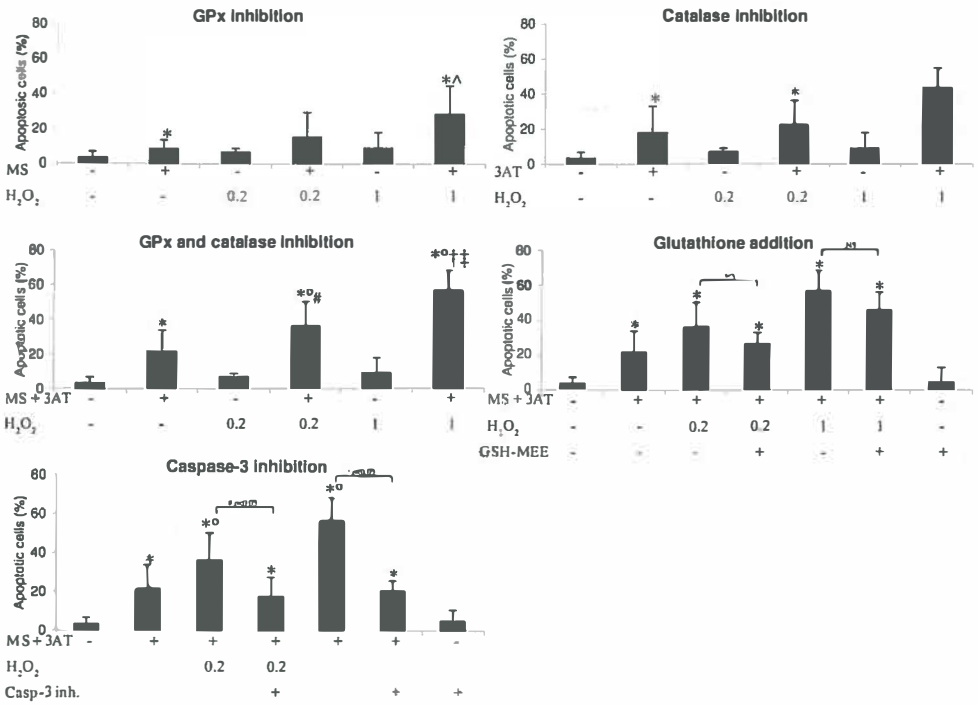

Figure 5. Inhibition of glutathione peroxidase and / or catalase induces apoptosis in activated hepatic stellate cells. A) Percentage of nuclei that were apoptotic after pre-treatment of HSCs with a GPx inhibitor (MS) and subsequent treatment with 0.2 or 1 mmol/L hydrogen peroxide. B) Percentage of nuclei that were apoptotic after pre-treatment of HSCs with a catalase inhibitor (3AT) and subsequent treatment with 0.2 or 1 mmol/L hydrogen peroxide. C) Inhibition of both Gpx and catalase induced high levels of apoptosis. D) Apoptosis induced by treatment with hydrogen peroxide in the presence of inhibitors of GPx and catalase is glutathione independent. Apoptosis induced by treatment with hydrogen peroxide in the presence of inhibitors of GPx and catalase is caspase-dependent. Results are shown as mean ± stdev of at least four independent experiments. *Significant difference compared to control, $p<0.05$; ^Significant difference compared to MS, $p<0.05$; §Significant difference compared to 3AT, $p<0.05$; † Significant difference compared to 1 mmol/L hydrogen peroxide, $p<0.05$; °Significant difference compared to MS + 3AT, $p<0.05$; #Significant difference compared to 0.2 mmol/L hydrogen peroxide, $p<0.05$; •Significant difference compared to 0.2 mmol/L hydrogen peroxide + 3-AT + MS, $p<0.05$; ‡Significant difference from 0.2 mmol/L hydrogen peroxide + 3-AT + MS, $p<0.05$; ¤Significant difference from 1 mmol/L hydrogen peroxide + 3-AT + MS, $p<0.05$.

Discussion

Chronic liver injury is almost invariably accompanied by increased oxidative stress, activation of stellate cells and fibrogenesis. Activated HSCs must be well protected against oxidative stress, since they survive and proliferate in the chronically injured liver. The oxidative stress in the chronically injured liver is composed of several reactive oxygen species, including hydrogen peroxide and superoxide anions. In this study we have investigated the resistance of hepatic stellate cells to hydrogen peroxide-induced injury. We demonstrate that this resistance is due, to a large extent, to a high intracellular glutathione content and increased expression of glutathione peroxidase in activated stellate cells. Although an increased glutathione content in activated stellate cells has been reported before (6), the implications have never been investigated in the context of cell death.

Maher et al. showed, in addition to increased glutathione levels upon activation, an increase in the activity and mRNA level of glutamate-cysteine ligase (GCL, a.k.a. γ-glutamylcysteine synthetase), the rate-limiting enzyme in glutathione synthesis (6). We did not observe an induction of GCL mRNA upon stellate cell activation in our own experiments. The increased cellular glutathione content is most likely due to a higher activity of GCL, especially since GCL activity is known to be regulated by the glutathione content (7).

Upon HSC activation, the expression of the hydrogen peroxide detoxifying enzyme glutathione peroxidase 1 (GPx1) is increased. This increase may be an adaptative response to oxidative stress. Indeed, mice over-expressing GPx1 are better protected against oxidative stress and they survive concentrations of the oxidant paraquat that are lethal in wildtype mice and even more harmful in GPx1 knockout mice (8). Mice that overexpress GPx1 are also more resistant to oxidative stress due to myocardial ischemia-reperfusion injury (9). It should be noted that glutathione is essential for the activity of GPx, since GPx converts reduced glutathione into oxidized glutathione. This might explain the coordinated increase in cellular glutathione content and GPx expression during the activation process of stellatec cells. Therefore, our data suggest that activated hepatic stellate cells may be more resistant against oxidative stress than quiescent stellate cells. Interestingly, upon activation the expression level of the mitochondrial superoxide anion converting enzyme Mn-SOD is strongly reduced. Disruption of the Mn-SOD gene, a known tumor suppressor gene, is lethal in mammalian organisms like the mouse. Lethality in these homozygous knockout mice is cause by impairment of mitochondrial function, leading to metabolic acidosis, ketosis and accumulation of lipids in the liver and skeletal muscle (10, 11). Characterization of the heterozygous Mn-SOD knockout mice revealed no compensatory increase of other ROS-detoxifying enzymes, like glutathione peroxidase, CuZn-SOD or catalase. Since Mn-SOD is restricted to the mitochondria, changes in its activity may not affect other components of the antioxidant defense system in other cellular compartment like the cytoplasm (12). At present it is not known how the activated stellate cells detoxify the reactive oxygen species generated in the mitochondria. The reduction in Mn-SOD mRNA expression during activation is most likely due to the strong reduction of the transcription factor peroxisome proliferator-activated receptor-γ (PPAR-γ) that occurs during stellate cell activation (data not shown and (13)). MnSOD expression is controlled by PPAR- γ: in PPAR-γ knockout mice, the expression of Mn-SOD is also reduced (14) and activation of PPAR-γ with the agonists rosiglitazone or rosuvastatin also enhanced MnSOD activity and expression (15;16).

Glutathione depletion has no effect on stellate cell morphology (17). In this study, we show that glutathione depletion also has no effect on the expression of markers of stellate cell activation, such as collagen type I, α-SMA and TGF-β or anti-oxidant genes like catalase, Mn-SOD, CuZn-SOD, and GPx1. Additionally, glutathione depletion did not affect the proliferation of hepatic

stellate cells. Therefore the loss of glutathione does not seem to be a key mechanism in the expression of the activated phenotype.

In contrast, increased mRNA levels of GCL and HO-1 were observed after glutathione depletion. Induction of GCL mRNA level after glutathione depletion has also been shown in lung epithelial cells (18), endothelial cells (19), and in vivo in rat liver (20). Although HO-1 is known to inhibit HSC proliferation via p38 activation (21), we did not find an alteration in the proliferation rate of stellate cells after HO-1 induction due to glutathione depletion. Possibly, the induction of HO-1 in our study by glutathione depletion is too modest to have an effect on p38 phophorylation and subsequent HSC proliferation. The induction of HO-1 by glutathione depletion is 2.5 times, whereas Li et al. (21, 22) showed at least a 10-fold induction of HO-1 expression mediated by 15-deoxy-delta-12,14-prostaglanding J2.

Although glutathione depletion alone had no effect on stellate cell viability, glutathione depletion increased the sensitivity of the cells to hydrogen peroxide-induced necrosis. Replenishing glutathione reduced necrotic cell death, without a shift towards apoptosis. Inhibition of the hydrogen peroxide detoxifying activity using inhibitors of glutathione peroxidase or catalase induced HSC apoptosis, both in the absence and in the presence of exogenous hydrogen peroxide. Apoptosis measured after inhibition of both glutathione peroxidase and catalase was shown to be independent of glutathione content but dependent on caspase-3 activity.

The observed difference in cell death, necrosis after glutathione depletion and apoptosis after inhibition of hydrogen peroxide detoxifying enzymes, might be explained by the cellular redox state. Glutathione is the most important regulator of the cellular redox state (23). Changes in the glutathione content will affect redox status and are known to influence activation of MAP Kinases, transcription factors and caspases (23-25). In the presence of glutathione, caspases that require reduced cysteine-sulfhydryl groups in their catalytic site, can still be activated when the enzymes GPx and catalase are inhibited. Inhibition of GPx and catalase is not likely to change the redox state of the cell. In contrast, in the absence of glutathione, e.g. after glutathione depletion, cells stimulated with hydrogen peroxide cannot activate caspases and the apoptotic programme and cell death is shifted towards necrosis. A shift from apoptotic to necrotic cell death has been reported before in hepatocytes (26).

In summary, our study reveals important changes in the defense against oxidative stress of hepatic stellate cells during activation. These changes are characterized by increased cellular glutathione content and GPx1 mRNA expression. Furthermore, we demonstrate that both glutathione and the hydrogen peroxide-converting enzymes GPx and catalase are important in the resistance against hydrogen peroxide-induced cell death. Our data suggests that activated hepatic stellate cells in vivo may acquire increased resistance to oxidative stress, providing an explanation for their survival in the fibrotic liver.

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Chapter 5

Ribavirin is anti-fibrotic in a non-viral rat model for liver fibrosis

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In preperation

Abstract

Background
Ribavirin, a nucleoside analogue, is used for treatment of hepatitis C virus (HCV) infection in combination with PEG-interferon-α (PEG-IFN-α). This co-treatment results in lower viral load and relapse rates, compared to interferon monotherapy. The anti-viral properties of both ribavirin and PEG-IFN-α have been extensively documented. PEG-IFN-α also has anti-fibrotic effects independent of viral clearance by inhibiting hepatic stellate cell (HSC) activation. In contrast, the effect of ribavirin on activated HSCs and liver fibrogenesis has never been studied. Ribavirin is a potent inhibitor of inosine-5'-monophosphate dehydrogenase (IMPDH). IMPDH inhibitors reduce proliferation in several cell types, including fibroblasts, mononuclear cells and leukaemia cells, and decrease plasma transforming growth factor-β (TGF-β) levels and renal fibrosis in nephrotoxic and immune-mediated kidney disease. Moreover, ribavirin monotherapy has been described to improve liver histology without reducing viral load.

Aim
To investigate whether ribavirin inhibits HSC proliferation in vitro and whether ribavirin reduces liver fibrosis markers in a non-viral in vivo model for liver fibrosis.

Methods
In vitro: Culture-activated rat HSCs were treated with ribavirin in the presence of 20% serum. Proliferation was measured using a BrdU incorporation assay. Apoptosis and necrosis were visualized by Acridine orange and Sytox green staining, respectively. In vivo: Bile duct ligation (BDL) was used as a model for liver fibrosis, using sham-operated controls. Rats were administered ribavirin (75 mg/kg/day) or vehicle for 10 days. Serum markers for liver injury (AST, ALT) and cholestasis (bilirubin) were measured. mRNA was isolated from total liver for real-time RT-PCR.

Results
Ribavirin dose-dependently inhibited serum-induced HSC proliferation (up to 80% reduction at 600 μmol/L), without inducing cell death. BDL-rats showed strongly induced mRNA expression of α-smooth muscle actin (α-SMA), TGF-β and collagen type 1 and increased serum markers for liver injury and cholestasis. Ribavirin-treated BDL-rats had significantly lower α-SMA (59% decrease), TGF-β (51% decrease) and collagen type 1 (63% decrease) mRNA levels than BDL-rats receiving vehicle, without change in animal weight or serum markers.

Conclusion
Ribavirin inhibits proliferation of activated HSCs in vitro and decreases mRNA markers for fibrosis in a non-viral in vivo model for liver fibrosis. We suggest that ribavirin may improve liver histology in HCV patients independent of viral clearance, by direct inhibition of HSC proliferation. Moreover, ribavirin may have therapeutic potential in non-viral fibrotic liver diseases.

Introduction

Over 170 million people worldwide are chronically infected with hepatitis C virus (HCV) (1). The majority of these patients develop liver fibrosis and eventually progress to liver cirrhosis. The mainstay of treatment for HCV-infected patients is PEG-interferon-α in combination with ribavirin (2). A sustained virological response of 40-60% is reached with this therapy (3, 4).
Ribavirin (1-beta-D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide) is a nucleoside analogue, first synthetized in 1972, with anti-viral properties against several RNA and DNA viruses both in vitro and in vivo (5). The predominant mechanism of action of ribavirin is still unclear (6). Ribavirin inhibits inosine-5'-monophosphate dehydrogenase (IMPDH) (7), thereby decreasing the availability of GTP for viral replication. Recently, Greupink et al. demonstrated that activated HSCs express IMPDH type 2 and that the IMPDH inhibitor mycophenolic acid (MPA) inhibits HSC proliferation in vitro (8). In addition, IMPDH inhibitors decrease proliferation in many other eukaryotic cell types and reduce fibrosis in kidney disease (9). Also, immunoregulatory actions have been described, e.g. induction of IFN-γ and IL-10 levels (10), and a Th2 to Th1 cell shift, which promotes the cellular immune response compared to the antibody-mediated immune response (11). Ribavirin can also be incorporated as a mutagenic nucleoside by viral RNA polymerase, creating lethal errors in viral RNA (12-14). However, Chevaliez et al. did not find an increase in HCV mutations in patients treated with ribavirin (15). The prevailing therapeutic mechanism of ribavirin in HCV patients is currently unknown.
Although ribavirin / PEG-interferon-α (PEG-IFN-α) co-treatment significantly improves the virological response compared to PEG-IFN-α monotherapy (16), ribavirin monotherapy does not affect the virological response in HCV patients (16). On the other hand, ribavirin monotherapy does decrease liver damage (decreased ALT levels) and improve liver histology (Metavir scores for activity and fibrosis) (16). We hypothesize that ribavirin has an anti-fibrotic effect independent from its anti-viral properties. Therefore we studied the effect of ribavirin on liver fibrosis in a non-viral model of fibrosis. Our study indicates that ribavirin inhibits hepatic stellate cell activation in vitro and is anti-fibrotic in vivo.

Materials and methods

Animals
Specified pathogen-free male Wistar rats were purchased from Charles River (Someren, Netherlands). They were housed under standard laboratory conditions with free access to standard laboratory chow and water. Each experiment was performed following the guidelines of the local committee for care and use of laboratory animals.

Bile duct ligation
Male Wistar rats (250-300g) were anesthetized and subjected to bile duct ligation (BDL, n=20) as a model of chronic cholestasis evolving into liver fibrosis (17). Ten days after BDL, rats were sacrificed, blood was collected and livers were perfused with saline and removed. Control rats (n = 4) received a sham operation (Sham).

Rat hepatic stellate cell isolation and culture
Hepatic stellate cells (HSCs) were isolated from male Wistar rats (500-600 g) by pronase and collagenase perfusion of the liver, followed by Nycodenz gradient (12% w/v) centrifugation as described previously (18).

Cells were then cultured in Iscove's Modified Dulbecco's Medium with Glutamax (Invitrogen, Breda, Netherlands) supplemented with 20% heat-inactivated fetal calf serum (Invitrogen), sodium-pyruvate (Invitrogen), non-essential amino acids (Invitrogen), 50 µg/mL gentamycine (Invitrogen) and Nystatin 250 U/mL (Sanofi-Synthelabo, Maassluis, Netherlands) in a humidified atmosphere containing 5% CO2 at 37°C. Primary HSC cultures were allowed to grow to confluence, subcultured by trypsinization and then cultured in Iscove's medium with supplements as described above, but without Nystatin.

Experimental design
Culture-activated HSC (passage 2-3) were used for all experiments, unless indicated otherwise. HSCs were exposed to the indicated concentrations of ribavirin.
10 BDL rats and 2 Sham rats received intragastric administration of ribavirin 75 mg/kg/day and 10 BDL rats and 2 Sham rats received only vehicle (saline) without ribavirin.

BrdU incorporation ELISA assay
Proliferation was measured using a BrdU incorporation ELISA assay (Roche Diagnostics Almere, the Netherlands) according to the manufacturer's instructions.

Analytical methods
Serum aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (AF), gamma-glutamyltransferase (γ-GT) and total bilirubin were determined by routine clinical chemistry.

RNA isolation, reverse-transcription-PCR and Real Time PCR
RNA was isolated using Tri Reagent (Sigma Aldrich) according to the manufacturer's instructions. RNA was quantified using the Ribogreen fluorescent assay (Invitrogen). Reverse transcription was performed on 1 µg of total RNA using random primers in a final volume of 50 µl. The reverse transcription program included 3 steps: 10 min. at 25°C followed by 1 hr at 37°C and 5 min at 95°C. For real-time PCR, 4 µl of 20x diluted cDNA was used in each PCR reaction in a final volume of 20 µl, containing 900 nmol/L of sense and antisense primers, 200 nmol/L of fluorogenic probe, 0.2 mmol/L dNTP's and 0.5 U Hot Goldstar Taq Polymerase (Eurogentec, Maastricht, Netherlands). 18S RNA was used as an internal control. Primers and probes used for real-time PCR are listed in Table 1.

Table 1. Oligonucleotide primers and probes used for Real time-PCR

Gene		Primers
18S	sense	5'-CGG CTA CCA CAT CCA AGG A-3'
	antisense	5'-CCA ATT ACA GGG CCT CGA AA-3'
	probe	5'-CGC GCA AAT TAC CCA CTC CCG A-3'
α-SMA	sense	5'-GCC AGT CGC CAT CAG GAA C-3'
	antisense	5'-CAC ACC AGA GCT GTG CTG TCT T-3'
	probe	5'-CTT CAC ACA TAG CTG GAG CAG CTT CTC GA-3'
TGF-β	sense	5'-GGG CTA CCA TGC CAA CTT CTG-3'
	antisense	5'-GAG GGC AAG GAC CTT GCT GTA-3'
	probe	5'-CCT GCC CCT ACA TTT GGA GCC TGG A-3'
Collagen type 1	sense	5'-TGG TGA ACG TGG TGT ACA AGG T-3'
	antisense	5'-CAG TAT CAC CCT TGG CAC CAT-3'
	probe	5'-TCC TGC TGG TCC CCG AGG AAA CA-3'

Acridine orange and Sytox green nuclear staining
Cells were seeded in 12-well plates and treated as indicated. Apoptosis was demonstrated by determining nuclear condensation assessed by acridine orange staining at 2.5 µg/mL (Sigma Aldrich). Necrosis was determined by Sytox Green nucleic acid staining at 0.5 µmol/L (Invitrogen). Cells were monitored over time using a Olympus CKX41 microscope.

Statistical analysis
Statistical analysis was performed using SPSS software, version 12.0. Results are expressed as the mean value and standard deviation. Statistical significance was calculated using a Student's t test, or a one-way ANOVA with Bonferroni post-hoc analysis for correction for multiple comparison. A corrected p value <0.05 was considered to be statistically significant.

Results

Ribavirin inhibits proliferation of activated hepatic stellate cells in a dose-dependent manner
Culture-activated rat hepatic stellate cells were exposed to 2.4 – 600 µmol/L ribavirin in the presence of 20% serum. Proliferation of HSCs dose-dependently decreased as measured by the BrdU incorporation ELISA. Significant reduction of proliferation was observed at concentrations of 150 µmol/L or higher (Figure 1).

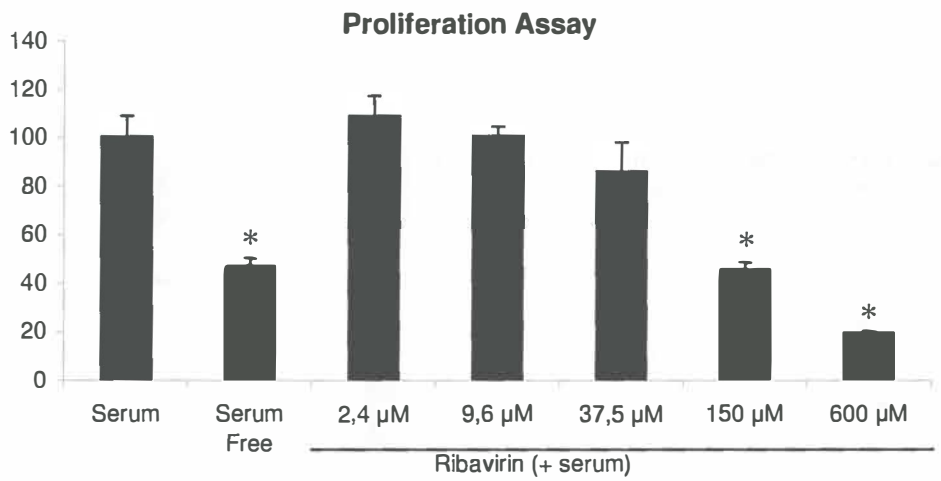

Figure 1. Ribavirin inhibits proliferation of activated hepatic stellate cells in a dose-dependent manner. Activated stellate cells were exposed to 2.4 - 600 µmol/L ribavirin in the presence of 20% serum. After 24 hours, BrdU was added for an additional 3 hours. Proliferation was estimated by measuring BrdU incorporation using an ELISA-based assay. Ribavirin dose-dependently inhibited serum-induced HSC proliferation.* $P < 0.05$ vs serum.

Ribavirin does not affect hepatic stellate cell viability
To investigate whether the anti-proliferative effect in Figure 1 was caused by cell death due to ribavirin toxicity, we stained HSCs with Acridine orange and Sytox green nuclear staining after exposure to 600 µmol/L ribavirin. Ribavirin did not induce apoptosis or necrosis at these concentrations (Figure 2 A,B).

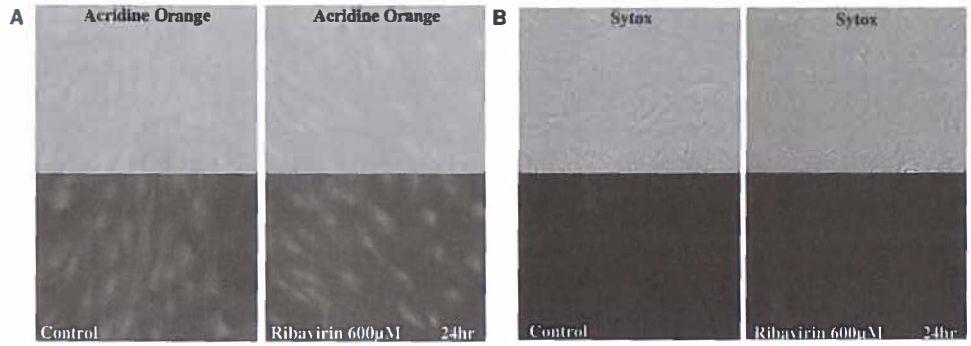

Figure 2. Ribavirin does not induce apoptosis or necrosis in activated stellate cells. Activated hepatic stellate cells were exposed to 600 μmol/L ribavirin for 24 hours in the presence of 20% serum. A) Ribavirin did not induce apoptosis as assessed by nuclear morphology using Acridine orange staining. B) Ribavirin did not induce necrosis as determined by Sytox green nuclear staining.

Ribavirin does not increase serum markers for cholestasis and liver injury in bile duct ligated rats

Serum markers for cholestasis and liver injury were assessed to rule out liver toxicity by ribavirin. Bile-duct ligated rats had increased levels of ALT, AST, AF, γ-GT and total bilirubin compared to sham-operated rats (Figure 3A-E). Ribavirin did not alter ALT, AST AF, γ-GT or total bilirubin levels.

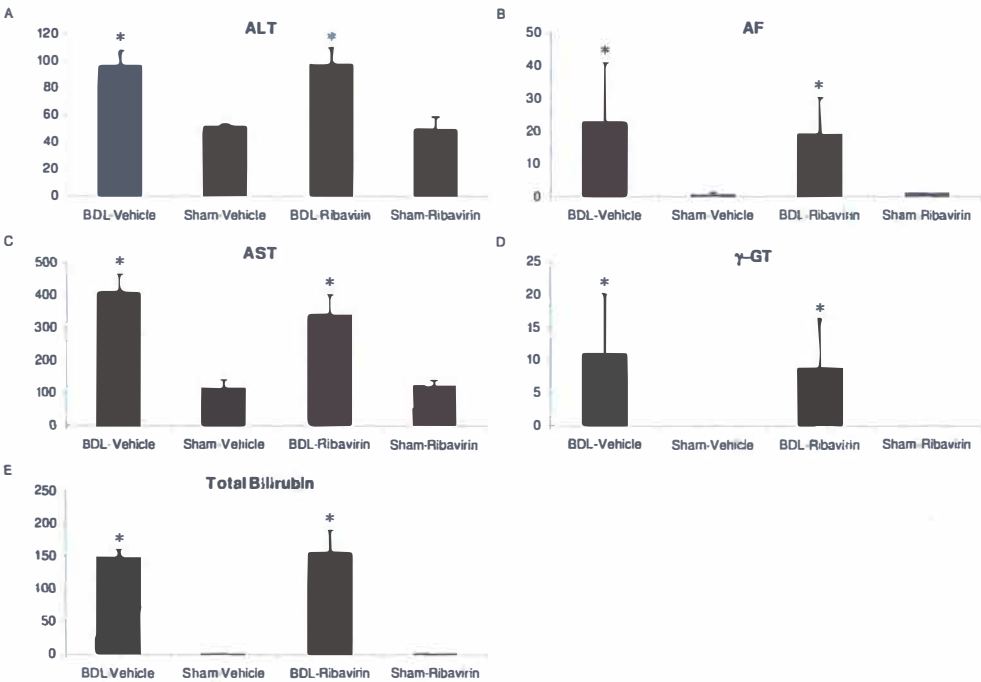

Figure 3. Ribavirin does not affect serum markers for cholestasis and liver injury in bile duct ligated rats. Serum markers for liver injury and cholestasis were measured. A-E) ALT, AST, AF, γ-GT and total bilirubin levels were increased in BDL-rats compared to sham-rats. Ribavirin treatment did not affect these markers. * $P < 0.05$ vs sham-vehicle and sham-ribavirin.

Ribavirin decreased mRNA expression of α-SMA, TGF-β, collagen type 1 in BDL rats
To study HSC activation and induction of fibrosis, we measured mRNA levels of α-SMA, TGF-β and collagen type 1. Total RNA was extracted from livers harvested 10 days after bile duct ligation or sham surgery. Liver tissue of BDL-rats demonstrated strongly induced mRNA levels of the fibrosis and HSC activation markers α-SMA, TGF-β and collagen type 1 compared to sham-operated rats. Ribavirin treatment reduced α-SMA (59% decrease), TGF-β (51% decrease) and collagen type 1 (63% decrease) mRNA levels in BDL-rats compared to BDL-rats receiving only vehicle (Figure 4A-C).

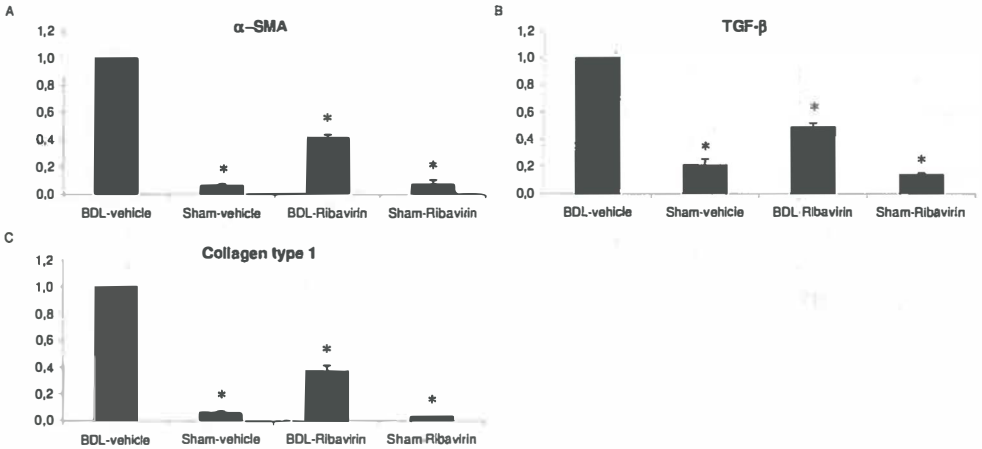

Figure 4. Ribavirin decreases mRNA expression of α-smooth muscle actin, TGF-β and collagen type 1 in bile duct ligated rats. mRNA levels of the HSC activation and fibrosis markers α-SMA, TGF-β and collagen type 1 were measured by quantitative real-time PCR. All markers were significantly increased in BDL-rats compared to sham-rats. Ribavirin treatment reduced mRNA levels of A) α-SMA by 59%, B) TGF-β by 51%, and C) collagen type 1 by 63%. * $P < 0.05$ vs BDL-vehicle.

Discussion

Ribavirin is a nucleoside analogue used in combination with PEG-IFN-α in the treatment of hepatitis C virus (HCV) infected patients. Since its mechanisms of action are still unclear, considerable research is performed to clarify its anti-viral properties. Ribavirin monotherapy improves markers of liver damage and liver histology without reducing viral load (16). Therefore, ribavirin may have an effect on fibrosis independent from its anti-viral actions.
The prodrug ribavirin is converted into ribavirin 5'-monophosphate (RMP) upon entering the cell, after which it is converted to ribavirin 5'-diphosphate (RDP) and –triphosphate (RTP). RMP is a competitive inosine-5'-monophosphate dehydrogenase (IMPDH) inhibitor, thereby decreasing GTP levels and GTP availability for viral replication. RTP inhibits RNA-dependent RNA polymerase and can be incorporated into viral RNA at the position of GTP, causing mutations (11, 19).
Greupink et al. demonstrated that activated HSCs express IMDPH type 2 and that the IMPDH inhibitor mycophenolic acid (MPA) inhibits HSC proliferation in vitro. Addition of exogenous guanosine to the culture medium abolished this effect (8). Moreover, the IMPDH inhibitors MPA, tiazofurin, and ribavirin have been reported to induce differentiation of the human prostate PC-3 tumor cell line towards prostate luminal cells (20). In vitro anti-proliferative effects of IMPDH

inhibitors have been described in renal tubular epithelium (21), mesangial cells (22), vascular smooth muscle cells (23), endothelial cells (24), fibroblasts (25-27) and myofibroblasts (28). In vivo, MPA was anti-proliferative and anti-fibrotic in immune-mediated and nephrotoxic kidney disease (29-32).

We hypothesized that ribavirin acts on hepatic stellate cells and that this may in part explain its anti-fibrotic effect in HCV patients. Moreover, if ribavirin is anti-fibrotic through a direct effect on hepatic stellate cells, ribavirin may act as an anti-fibrotic agent in liver fibrosis in general. Therefore, we tested whether ribavirin could reduce liver fibrosis in bile duct ligated rats, a non-viral model of liver fibrosis.

Ribavirin dose-dependently decreased serum-induced hepatic stellate cell proliferation in vitro, without causing apoptosis or necrosis. In bile duct ligated rats, 10 days postoperatively, no effect of ribavirin was observed on serum markers for liver damage. However, total liver RNA isolates showed significantly decreased levels of the fibrosis markers α-SMA, TGF-β and collagen type I.

The mechanism of action of ribavirin in the treatment of HCV may not be exclusively due to its anti-viral properties, but may also be due to a direct anti-proliferative (and hence anti-fibrotic) effect on hepatic stellate cells. We suggest that ribavirin is not only suitable for treatment of hepatitis C infection, but may also be considered in the treatment of other chronic liver diseases that are characterized by stellate cell activation and proliferation.

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Chapter 6

Summary and general discussion

Future perspectives

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Summary and general discussion

In the normal liver, the hepatic stellate cell has a quiescent (i.e. non-proliferating) phenotype. It is the main storage site for vitamin A (retinoids) and it produces the appropriate quality and quantity of extracellular matrix. In chronic liver injury, a sustained wound healing response takes place in the liver. This sustained wound healing response is characterized by a phenotypic transformation of the stellate cell from a quiescent phenotype to an "activated" phenotype. This activated phenotype is characterized by loss of retinoids, increased proliferation, increased production of extracellular matrix (ECM), in particular fibrillar collagens, and increased responsiveness to growth factors, cytokines and chemokines. The replacement of normal low density ECM by high density fibrillar collagen-rich ECM contributes to the capillarization of the sinusoids and the replacement of functional liver cells (hepatocytes) by scar tissue. Together, these changes may lead to portal hypertension and loss of liver function. Cirrhosis, the advanced stage of liver fibrosis, is frequently accompanied by the development of liver tumors and hence the prognosis for patients with liver cirrhosis is poor (1-3).
It has proven to be extremely difficult to effectively treat liver fibrosis. Other than removing the causative agent (alcohol, hepatitis B or C virus), there are no treatment options. For many patients, liver transplantation is the only effective treatment. However, this option is complicated and not without risk due to the shortage of donor livers, peri- and postoperative mortality and, in case of viral hepatitis, re-infection of the donor liver by the virus.
Although the stellate cell has been identified as a key player in fibrogenesis, current treatments are not directed towards stellate cells. Some drugs that affect stellate cell proliferation or viability have been used in experimental models of liver fibrosis, e.g. pentoxifylline (4, 5) and gliotoxin (6), but human trials are (still) lacking. The activated stellate cell is an attractive target for intervention, since activation of stellate cells is a common feature of all chronic liver diseases regardless of their etiology. Therefore, any therapy aimed at the stellate cell could in principle be used for a wide variety of chronic liver diseases. These therapies should reduce the number of activated stellate cells, either by reversing the activated phenotype into the quiescent phenotype, or by inducing cell death of the activated stellate cell.
A paradox emerges from the study of chronically injured livers: in these livers all cell types are present in the same "intrahepatic" environment and all cell types are exposed to the same cocktail of bile acids, toxic reactive oxygen species and apoptotic cytokines (e.g. tumor necrosis factor and Fas-ligand). Yet, whereas the hepatocytes, the functional parenchymal liver cells, perish in these conditions, the stellate cells become activated and proliferate. Also the Kupffer cells, the resident macrophages of the liver, become activated and synthesize increased amounts of cytokines. This is not only a paradox (one cell type perishes, the other thrives), it also points towards a strategy for the development of interventions that aim to reduce the number of activated stellate cells.
The elucidation of the mechanisms that explain the unusual resistance to cell death of activated stellate cells and their survival in the chronically injured liver may yield strategies to interfere with these survival mechanisms. Therefore, the major goal of the research described in this thesis was to uncover the survival mechanisms of activated stellate cells.

Chapter 2 describes the expression, regulation and function of Mdr- and Mrp-type transporters in culture-activated hepatic stellate cells. The rationale for this study was the observation that certain cell types survive and proliferate in hostile environments due to the expression of specific Mdr/Mrp-type transporters. These transporters export potentially toxic metabolites out of the cell. Such a phenomenon has been observed in cancer cells that very often express

high levels of Mdr/Mrp-type-transporters (7, 8). Likewise, the multi-drug resistant phenotype is also expressed in progenitor cells (9, 10). These are precursor cells that expand into cells of a specific lineage to restore the number of these cells. We have previously shown that, within the liver in conditions of massive liver cell death (e.g. acute liver failure), the hepatic progenitor cell compartment is activated and expands, restoring the functional liver mass (9). In addition, we have shown that the expansion of this population is associated with (increased) expression of Mdr- and Mrp-type transporters (9, 10). The presence, regulation and function of Mdr- and Mrp-type transporters in (activated) stellate cells have never been investigated before. In **chapter 2** we demonstrate that activated rat HSCs express Mrp1, Mrp3, Mrp4, Mdr1a and Mdr1b mRNA. This is a similar expression pattern as observed in hepatic progenitor cells (9). Although these transporters are not regulated by oxidative stress and only to some extent by cytokines, we show that these transporters, in particular Mrp1, are important for the survival of activated stellate cells. An important follow-up of this research would be to apply models of liver fibrosis to Mrp-specific knockout mice, in particular Mrp1. So far, no studies investigating liver fibrosis in Mrp-knockout mice have been performed. Interestingly, a recent report suggested a relationship between stellate cells and progenitor cells, by showing that CD133-positive hepatic stellate cells could serve as precursors of several liver cell types, including hepatocytes and endothelial cells (11). It should be noted that a therapy aimed at blocking Mrp1 function will be complicated by the fact that in chronic liver diseases, the progenitor cell compartment is also activated and probably has a role in restoration and/or maintenance of the number of functional cells. Therefore, the use of an Mrp1 blocker in the treatment of fibrosis might actually inhibit this beneficial expansion of the progenitor cell population.

In **chapter 3 and 4** we investigate in detail the response of hepatic stellate cells to oxidative stress and the defense mechanisms involved in the protection against oxidative stress. The rationale for these studies is that it is well established that liver fibrosis is positively correlated with oxidative stress. Chronic liver injury, evolving into liver fibrosis is invariably accompanied by oxidative stress (12). Anti-oxidants, at least in experimental models, have shown some beneficial effects on liver fibrosis, although there remains controversy (13, 14). Several studies have reported direct effects of reactive oxygen species on aspects of stellate cell activation (15, 16), including proliferation, opposite effects of ROS have also been reported (17, 18). Since many of these studies have investigated the effect of ROS on only one aspect of stellate cell biology, we performed a systematic evaluation of the effects of two reactive oxygen species on several aspects of stellate cell activation (activation markers, proliferation) and viability (apoptotic and necrotic cell death) in one comprehensive study. In **chapter 3**, we investigated the effects of ROS on stellate cell proliferation and cell death. We report that both hydrogen peroxide and superoxide anions (menadione) do not directly stimulate stellate cell proliferation. In fact, these ROS inhibit stellate cell proliferation induced by either serum or platelet-derived growth factor (PDGF). Furthermore, we demonstrate that hydrogen peroxide does not induce cell death of stellate cells, indicating that stellate cells are unusually resistant to hydrogen peroxide induced toxicity. Only at supraphysiological concentrations of 5 mmol/L or higher does hydrogen peroxide induce necrotic cell death. Superoxide anions (generated by the redox-cycling of menadione) induce apoptotic cell death. Interestingly, this menadione-induced apoptosis is caspase-independent. Caspase-independent ROS-induced apoptosis has been reported before (19, 20). Apoptotic cell death, independent of caspases may be effectuated by granzymes (21).

In contrast, we have previously shown that menadione–induced apoptosis of hepatocytes is caspase-dependent. Therefore, the involvement of caspases in superoxide anion induced apoptosis is cell type specific. This is an interesting observation, since it will also affect the effectiveness of caspase-inhibitors in ROS-mediated liver injury. Another interesting observation is the differential effect of hydrogen peroxide and superoxide anions on cellular glutathione levels of activated stellate cells. Whereas hydrogen peroxide has no effect on the cellular glutathione level, menadione dose-dependently reduced glutathione content. A possible explanation is that menadione is able to interact with reduced glutathione forming a menadione-GSH conjugate, while at the same time still being able to produce superoxide anions (22). The reported decrease in glutathione could then be due the export of this conjugate. In contrast, conjugation of hydrogen peroxide to GSH is unknown. Finally, we demonstrate that menadione-induced apoptosis can be reversed by restoring glutathione levels. This observation is very important since it identifies cellular glutathione content as an important determinant in the resistance against superoxide anion-induced cell death of stellate cells. This is in line with the observation that loss of glutathione, via Mrpl is a necessary signal for apoptosis in Jurkat cells (23). Therefore, the use of anti-oxidants in the treatment of liver fibrosis could have, as undesirable side-effect, the improvement of stellate cell resistance against cell death. This calls for selectivity in the application of anti-oxidants (or glutathione restoring interventions).

Chapter 4 extends the studies of **chapter 3**. In **chapter 4** we investigate the importance of anti-oxidant defense mechanisms in stellate cells. The rationale for this study was that the resistance against oxidative stress-induced toxicity might stem from a powerful anti-oxidant defense system, including anti-oxidants (e.g. glutathione) and ROS-detoxifying enzymes like catalase or glutathione peroxidases. Furthermore, we speculate that activated stellate cells have a different anti-oxidant potential than quiescent stellate cells. Again, this is a topic that has never been investigated in hepatic stellate cells. In **chapter 4** we demonstrate that activation of stellate cells is accompanied by profound changes in anti-oxidant defense systems. First of all, the cellular glutathione pool is 5-6 times increased in activated stellate cells compared to quiescent cells. Secondly, the expression of the hydrogen peroxide detoxifying enzyme glutathione peroxidase 1 (GPx1) is highly induced, whereas catalase expression remains unchanged. We next demonstrated that glutathione is important in the protection against hydrogen peroxide induced toxicity: depletion of glutathione dramatically increased the sensitivity of activated stellate cells to hydrogen peroxide-induced necrosis. In contrast, the inhibition of the hydrogen peroxide detoxifying enzymes GPx and catalase increased apoptotic death of activated stellate cells. This apparent paradox (apoptotic vs necrotic cell death induced by hydrogen peroxide) could be explained by differences in the glutathione-status in these conditions. Hydrogen peroxide-induced stellate cell apoptosis is dependent on caspase-activity. Caspases are redox-sensitive enzymes that require reduced sulfhydryl groups of cysteine residues in their cataltytic site (24, 25). In the case of glutathione depletion, caspase activation may be limited due to the absence of reduced cys-SH groups. In the case of inhibition of GPx and catalase, glutathione levels are sufficient to permit caspase activation. This is supported by the finding that glutathione supplementation did not affect hydrogen peroxide-induced apoptosis induced by inhibitors of catalase and GPx. An interesting finding in our study was that stellate cell activation is accompanied by a dramatic reduction in the expression of the superoxide anion converting enzyme Mn-superoxide dismutase (Mn-SOD). This was unexpected: Mn-SOD is responsible for the detoxification of superoxide anions generated in the mitochondria. A Mn-SOD deficient genotype results in a lethal phenotype indicating the importance of this enzyme for normal cellular metabolism and viability (26, 27). How stellate cells survive the low level of Mn-SOD expression is not clear at this point. There is no compensatory increase in the cytoplasmic Cu,Zn-

SOD. Furthermore, our data need to be confirmed at the protein level. A likely explanation for the reduction of Mn-SOD is the reduction of the transcription factor PPAR-γ in activated stellate cells (28). Several reports indicate that Mn-SOD expression is controlled by this transcription factor (29-31). Whether the reduction of Mn-SOD is (partly) responsible for the acquisition of the activated phenotype is currently not known: an interesting follow-up study would be to investigate the effect of overexpressing Mn-SOD in activated stellate cells: will the stellate cells revert to the quiescent phenotype?

It is important to note that glutathione itself does not govern the activation of stellate cells: depletion of glutathione does not affect morphology, cell death, proliferation or markers of stellate cell activation. Likewise, restoration of glutathione content did not change morphology, cell death, and proliferation. Glutathione is the most important regulator of the cellular redox state (32). On the other hand, changes in redox status may influence activation of numerous kinases, including MAP Kinases and transcription. Therefore, it is very likely that changes in the cellular glutathione content do affect several functions of stellate cells (32-34).

A very obvious strategy to treat liver fibrosis would be to inhibit the proliferation of activated stellate cells. In fact, some experimental drugs have been used that inhibit stellate cell proliferation, e.g pentoxifylline. Inhibitors of proliferation specifically aimed at the stellate cell have not been used in human trials yet. However, inhibitors of viral proliferation (DNA polymerase inhibitors) are used in viral liver diseases. The standard treatment for hepatitis C virus infection is the combination of pegylated interferon-α2b and the viral DNA-polymerase inhibitor ribavirin, a nucleoside analogue. In **Chapter 5** we have investigated the effect of the nucleoside analogue ribavirin in a non-viral model of liver fibrosis. The rationale for this study was that the viral DNA-polymerase inhibitor might also have effects on the proliferation of rapidly dividing eukaryotic cells, e,g activated stellate cells. Ribavirin is a competitive inhibitor of inosine-5'-monophosphate dehydrogenase (IMPDH). IMPDH inhibitors reduce proliferation of several eukaryotic cell types including renal tubular epithelium (35), mesangial cells (36), vascular smooth muscle cells (37), endothelial cells (38), fibroblasts (39-41), myofibroblasts (42). In vivo, IMPDH inhibitors are anti-proliferative and anti-fibrotic in immune-mediated and nephrotoxic kidney disease (43-46).

We first demonstrated that ribavirin inhibits the proliferation of activated stellate cells in vitro in a dose-dependent manner, without inducing apoptotic or necrotic cell death of the stellate cell. Next, we used a non-viral model of liver fibrosis in the rat, the bile duct ligation model. This model leads to overt signs of fibrosis within 10 days. Daily administration of ribavirin reduced several markers of fibrosis and stellate cell activation, including reduced expression of the stellate cell activation markers α-smooth muscle actin and TGF-β and the extracellular matrix protein collagen type I. Important questions that remain to be answered are the long term effects of ribavirin treatment and the effect of ribavirin in the reversal or slow down of already established fibrosis. In addition, it is important to determine whether the observed changes in gene expression are also reflected in corresponding changes in protein levels (matrix deposition, number of activated stellate cells). Our observations may also provide an explanation for the observation that in some patients with hepatitis C virus infection, treated with ribavirin, there is an improvement in the histology and the inflammation score, despite a failure to eradicate the virus completely (47). Our data suggest that ribavirin might be beneficial in the treatment of non-viral liver diseases.

Future perspectives

In this thesis we have addressed the protective mechanisms of activated hepatic stellate cells against oxidative stress-induced toxicity. We have uncovered important novel information with regard to the role of anti-oxidants, ROS-detoxifying enzymes, glutathione, and Mdr- and Mrp-type transporters in the protection against oxidative stress. Several of these observations deserve follow up. Interesting novel targets for intervention resulting from this study are Mrp1, glutathione content and ribavirin. However, it should be realized that these interventions should be targeted to the right cell type. Many interventions that will cripple the stellate cell will also cripple the hepatocyte or progenitor cells, which is clearly not desirable. In this respect, the emerging knowledge about targeting drugs specifically to stellate cells is very important (48). A "stellate cell" based intervention also has the advantage that all liver diseases evolving into fibrosis can be addressed.

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Chapter 7

Nederlandse samenvatting en discussie
Toekomst perspectief

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Nederlandse samenvatting en discussie

In de normale lever heeft de lever stellaatcel een "rustend" (d.w.z. niet prolifererend) fenotype. De stellaatcel is de voornaamste opslagplaats voor vitamine A (retinoiden) en produceert de juiste kwaliteit en kwantiteit extracellulaire matrix (ECM). Tijdens chronische leverschade vindt er een aanhoudende wondhelingrespons plaats in de lever. Deze aanhoudende wondhelingrespons wordt gekarakteriseerd door een fenotypische transformatie van de stellaatcel van een "rustend" (quiescent) fenotype naar een "geactiveerd" fenotype. Dit actieve fenotype wordt gekenmerkt door verlies van vitamine A, verhoogde proliferatie, verhoogde productie van ECM (in het bijzonder fibrillaire collagenen) en verhoogde response op groeifactoren, cytokinen en chemokinen.
De vervanging van normaal ECM met een lage dichtheid door ECM met een hoge dichtheid draagt bij aan de capillarisatie van het sinusoid en het vervangen van functionele levercellen (hepatocyten) door littekenweefsel. Gezamenlijk kunnen deze veranderingen zorgen voor portale hypertensie en het verlies van leverfunctie. Cirrose, het gevorderde stadium van leverfibrose, gaat vaak samen met de ontwikkeling van levertumoren en hierdoor is de prognose voor patiënten met levercirrose slecht.
Het is extreem moeilijk om leverfibrose effectief te behandelen. Behandelmethoden zijn erop gericht om de veroorzaker van de leverschade (alcohol, hepatitis B of C virus) te verwijderen, verder zijn er geen behandelmethoden. Voor veel patiënten is levertransplantatie de enige effectieve behandeling. Deze optie wordt gecompliceerd door een tekort aan leverdonoren, pre- en postoperatieve sterfte en in het geval van virale hepatitis, door re-infectie van de donorlever met het virus.
Hoewel de stellaatcel is geïdentificeerd als de belangrijkste speler in de ontwikkeling van leverfibrose, zijn de huidige behandelmethoden niet gericht op de stellaatcel. Sommige medicijnen die effecten hebben op de proliferatie en viabiliteit van de stellaatcel zijn gebruikt in experimentele modellen van leverfibrose, bijvoorbeeld pentoxifylline en gliotoxine, maar nog niet in studies met patiënten. De geactiveerde stellaatcel is een aantrekkelijk doelwit, omdat activatie van de stellaatcel een kenmerk is van alle chronische leverziekten, ongeacht de achterliggende oorzaak. Daarom zou elke therapie die gericht is op de stellaatcel in principe gebruikt kunnen worden voor behandeling van een breed scala aan chronische leverziekten. Deze therapieën moeten het aantal geactiveerde stellaatcellen reduceren, door het actieve fenotype terug te laten keren naar het rustende fenotype of door het induceren van celdood van geactiveerde stellaatcellen.
Een paradox doet zich voor in de studie van chronisch beschadigde levers: in deze levers worden alle leverceltypen blootgesteld aan dezelfde cocktail van toxische galzouten, reactive zuurstof verbindingen en apoptotische cytokinen (zoals tumor necrosis factor en Fas-ligand). Maar de hepatocyt, de functionele levercel, gaan verloren onder deze omstandigheden, terwijl de stellaatcel activeert en prolifereert. Ook de Kupffer cellen, de macrofagen van de lever, worden geactiveerd en produceren meer cytokinen. Dit is niet alleen een paradox (één celtype gaat verloren, terwijl het andere gedijt), het pleit ook voor de ontwikkeling van een strategie van interventie welke gericht is op het verminderen van het aantal geactiveerde stellaatcellen.
De opheldering van de mechanismen van geactiveerde stellaatcellen die de ongebruikelijke resistentie tegen celdood en hun overleving in de chronisch beschadigde levers verklaren, kunnen strategieën opleveren om te interfereren in deze overlevingsmechanismen. Daarom was het belangrijkste doel van dit proefschrift het ontrafelen van de overlevingsmechanismen van geactiveerde stellaatcellen.

Hoofdstuk 2 beschrijft de expressie, regulatie en functie van Mdr- en Mrp-transporters in in vitro geactiveerde lever stellaatcellen. De reden voor deze studie is de observatie dat bepaalde

celtypen overleven en prolifereren in een "vijandelijke" omgeving als gevolg van de expressie van specifieke Mdr/Mrp-transporters. Deze transporters exporteren toxische metabolieten de cel uit. Dit fenomeen is ook waargenomen in kankercellen die vaak een hoge expressie van Mdr/Mrp-transporters hebben. Het multi-drug resistente fenotype wordt ook tot expressie gebracht in progenitor cellen. Dit zijn precursor cellen die kunnen differentiëren in specifieke cellen ter compensatie van het verlies van deze cellen. We hebben in het verleden aangetoond dat in de lever in condities van massale leverceldood (d.w.z. acuut leverfalen), het progenitor celcompartiment wordt geactiveerd en expandeert waarbij het de functionele lever massa doet toenemen. Tevens hebben we aangetoond dat deze populatie wordt geassocieerd met (verhoogde) expressie van Mdr- en Mrp-transporters. De aanwezigheid, regulatie en functie van Mdr- and Mrp-transporters in (geactiveerde) stellaatcellen is nog niet eerder onderzocht. In **hoofdstuk 2** tonen we aan dat geactiveerde rat stellaatcellen mRNA van Mrpl, Mrp3, Mrp4, Mdrla en Mdrlb tot expressie brengen. Een vergelijkbaar patroon is waargenomen in progenitor cellen. Hoewel deze transporters niet gereguleerd worden door oxidatieve stress en enigszins gereguleerd worden door cytokinen, tonen we wel aan dat deze transporters, in het bijzonder Mrpl, belangrijk zijn voor de overleving van geactiveerde stellaatcellen. Een interessante vervolgstudie van dit onderzoek zou zijn om leverfibrose modellen toe te passen op Mrp-specifieke knock-out muizen, vooral op Mrpl knock-out muizen. Tot nu toe zijn er geen studies naar leverfibrose in Mrp- knock-out muizen gedaan. Interessant is een recent verschenen artikel dat een relatie tussen progenitor cellen en stellaatcellen suggereert. Dit artikel laat zien dat CDl33-positieve lever stellaatcellen kunnen dienen als voorlopers van verschillende celtypen, waaronder hepatocyten en endotheel cellen. Een therapie gericht op het blokkeren van de functie van Mrpl zal gecompliceerd worden door het feit dat in chronische leverziekten het progenitor celcompartiment ook geactiveerd is en een rol heeft in herstel en/of onderhoud van het aantal functionele cellen. Daarom zal het gebruik van een Mrpl blokker als behandeling voor fibrose misschien het voordeel van de expansie van de progenitor celpopulatie te niet doen.

In **hoofdstuk 3 en 4** hebben we in detail onderzocht wat de response is van stellaatcellen op oxidatieve stress en het defensiemechanisme dat betrokken is bij de bescherming tegen oxidatieve stress. De reden voor deze studie is de positieve correlatie tussen leverfibrose en oxidatieve stress. Chronische leverschade, resulterend in leverfibrose, gaat altijd gepaard met oxidatieve stress. Anti-oxidanten hebben in experimentele modellen enige positieve effecten gehad op leverfibrose, maar er blijft controverse bestaan. Verschillende studies hebben directe effecten van reactieve zuurstofverbindingen (ROS) op stellaatcelactivatie gerapporteerd, inclusief proliferatie. Echter, tegenovergestelde effecten van ROS zijn ook gerapporteerd. Omdat veel van deze studies naar de effecten van ROS slecht één aspect van stellaatcelbiologie belichten, hebben wij een systematische evaluatie uitgevoerd van de effecten van twee reactieve zuurstofverbindingen op verschillende aspecten van stellaatcelactivatie (activatiemerkers, proliferatie) en viabiliteit (apoptotische en necrotische celdood) in één veelomvattende studie. In **hoofdstuk 3** hebben we de effecten van ROS op stellaatcelproliferatie en celdood onderzocht. Wij hebben aangetoond dat zowel waterstofperoxide als superoxide anionen (menadione) niet direct stellaatcelproliferatie stimuleren. Eigenlijk remmen deze reactieve zuurstofverbindingen juist serum of groeifactor (platelet-derived growth factor: PDGF) geïnduceerde proliferatie. Daarnaast hebben we aangetoond dat waterstofperoxide geen celdood induceert van stellaatcellen, hetgeen aantoont dat deze cellen buitengewoon resistent zijn tegen waterstofperoxide geïnduceerde toxiciteit. Alleen bij zeer hoge concentraties van 5 mmol/L of hoger, induceert waterstofperoxide necrotische celdood. Superoxide anionen (gegenereerd door redox-cycling van menadione) induceert apoptotische celdood. Deze menadione geïnduceerde apoptose is, interessant genoeg, caspase

onafhankelijk. Caspase onafhankelijk ROS-geïnduceerde apoptose is eerder gerapporteerd en kan mogelijk veroorzaakt worden door granzymes.
Deze bevinding verschilt van onze eerdere bevinding, dat menadione geïnduceerde apoptose in hepatocyten caspase afhankelijk is. Hieruit blijkt dat er celspecifieke verschillen zijn in de mechanismen van superoxide anion-geïnduceerde apoptose. Dit is een interessante observatie, omdat het de effectiviteit van caspase-inhibitors in ROS-gemedieerde leverschade zal beïnvloeden. Een andere interessante observatie is het verschil tussen waterstofperoxide en superoxide anionen op de cellulaire glutathione gehaltes. Waterstofperoxide heeft geen effect op de cellulaire glutathione gehaltes, terwijl menadione dosisafhankelijk de glutathione gehaltes reduceert. Een mogelijke verklaring hiervoor is dat menadione een interactie kan aangaan met glutathione waarbij het menadione-GSH conjugaat ontstaat, waarbij het nog steeds superoxide anionen kan blijven produceren. De gerapporteerde afname van glutathione wordt dan veroorzaakt door de export van het gevormde conjugaat. Conjugatie van waterstofperoxide met glutathione is onbekend. Ten slotte laten we zien dat menadione-geïnduceerde apoptose kan worden tegengegaan door de glutathione gehaltes te herstellen. Deze observatie is erg belangrijk, omdat het cellulair glutathione identificeert als een belangrijk component in de resistentie tegen superoxide anion-geïnduceerde celdood van stellaatcellen. Deze observatie komt overeen met de bevinding dat verlies van glutathione, via Mrp1, een benodigd signaal is voor apoptose in Jurkat cellen. Het gebruik van anti-oxidanten in de behandeling van leverfibrose kan ongewenste bijeffecten hebben, namelijk het verbeteren van stellaatcel resistentie tegen celdood. Dit geeft aan dat selectiviteit geboden is bij het toepassen van anti-oxidanten (of interventies die glutathione gehaltes herstellen).

Hoofstuk 4 is een uitbreiding van de studie van **hoofdstuk 3**. In **hoofdstuk 4** bestuderen we het belang van anti-oxidant beschermingsmechanismen in stellaatcellen. De reden voor deze studie is dat de resistentie tegen oxidatieve stress-geïnduceerde toxiciteit mogelijk wordt verkregen door een krachtig anti-oxidant beschermingsysteem, waaronder anti-oxidanten (zoals glutathione) en ROS-detoxificerende enzymen (zoals catalase en glutathione peroxidase). Daarnaast speculeren wij dat gactiveerde stellaatcellen een ander anti-oxidant fenotype hebben dan rustende stellaatcellen. Ook dit is nog niet eerder onderzocht in lever stellaatcellen.
In **hoofdstuk 4** laten we zien dat activatie van stellaatcellen gepaard gaat met grote veranderingen in het anti-oxidant fenotype. In de eerste plaats is de glutathione concentratie 5 tot 6 keer verhoogd in geactiveerde stellaatcellen ten opzichte van rustende stellaatcellen. Ten tweede is de expressie van het waterstofperoxide detoxificeerde enzym glutathione peroxidase 1 (GPx1) sterk verhoogd, terwijl de catalase expressie gelijk blijft. Vervolgens tonen we aan dat glutathione belangrijk is in de bescherming tegen waterstofperoxide: depletie van glutathione verhoogt de gevoeligheid van geactiveerde stellaatcellen voor waterstofperoxide geïnduceerde necrose. Remming van de waterstofperoxide detoxicificerende enzymen catalase en GPx induceert echter apoptose van geactiveerde stellaatcellen. Dit lijkt paradoxaal (apoptose tegenover necrose geïnduceerd door waterstofperoxide), maar dit kan worden verklaard door de verschillen in de glutathione status in deze condities. Waterstofperoxide geïnduceerde apoptose is afhankelijk van caspase-activiteit. Caspases zijn redox-gevoelige enzymen die een gereduceerde sulfhydryl groep van cysteïne residuen nodig hebben voor hun caspase-activiteit. In het geval van remming van GPx en catalase zijn de glutathione levels toereikend om caspase activiteit toe te staan. Dit wordt ondersteund door de bevinding dat glutathione suppletie geen effect heeft op de watersofsperoxide-geïnduceerde apoptose na remming van catalase en GPx. Een interessante bevinding van onze studie is de dramatisch verlaagde expressie van het superoxide anion omzettend enzym Mn-superoxide dismutase (Mn-SOD). Dit was een onverwachte bevinding:

Mn-SOD is verantwoordelijk voor de detoxificatie van superoxide anionen gegenereerd in de mitochondrieën. Een Mn-SOD deficiënt genotype resulteert in een lethaal fenotype. Dit geeft aan dat dit enzym erg belangrijk is in het normale cellulaire metabolisme. Hoe stellaatcellen dit lage expressie niveau van Mn-SOD overleven is op dit moment nog niet duidelijk. Er is geen compensatie door verhoging van het cytoplasmatische Cu,Zn-SOD. Deze bevindingen moeten nog worden bevestigd op eiwitniveau. Een aannemelijke verklaring voor de verlaging van Mn-SOD is de reductie van de transcriptiefactor PPAR-γ in geactiveerde stellaatcellen. Verschillende artikelen wijzen erop dat Mn-SOD expressie wordt gecontroleerd door deze transcriptiefactor. Of de reductie van Mn-SOD (deels) verantwoordelijk is voor de verwerving van het geactiveerde fenotype is onbekend: een interessante vervolgstudie zou zijn om het effect van overexpressie van Mn-SOD in geactiveerde stellaatcellen te bestuderen. Zullen de stellaatcellen dan terugkeren naar een rustend fenotype?
Het is belangrijk om op te merken dat glutathione zelf niet de oorzaak is van activatie van stellaatcellen: depletie van glutathione heeft geen effect op morfologie, celdood, proliferatie of activatie merkers van stellaatcellen. Ook het herstellen van de glutathione status heeft geen effect op morfologie, celdood en proliferatie. Glutathione is de belangrijkste regulator van de cellulaire redox staat. Veranderingen in de redox staat kunnen invloed hebben op de activiteit van meerder kinases, inclusief de MAP-kinases en transcriptiefactoren. Daarom is het aannemelijk dat veranderingen in de cellulaire glutathione status verschillende functies van de stellaatcel beïnvloeden.

Een voor de hand liggende strategie zou zijn om leverfibrose te behandelen door de proliferatie van stellaatcellen te voorkomen. Sommige experimentele medicijnen zijn gebruikt om stellaatcel proliferatie te voorkomen, bijvoorbeeld pentoxifylline. Remmers van proliferatie die specifiek gericht zijn op de stellaatcel worden (nog) niet gebruikt in klinische trials. Echter, remmers van virale proliferatie (DNA polymerase remmers) worden gebruikt voor de behandeling van virale leverziekten. De standaardbehandeling voor een hepatitis C virus infectie is een combinatie van gepegyleerd interferon-α2b en de virale DNA-polymerase remmer ribavirine, een nucleoside analoog. In **hoofdstuk 5** hebben we de effecten van de nucleoside analoog ribavirine onderzocht in een niet-viraal model van leverfibrose. De reden voor deze studie is dat de virale DNA-polymerase remmer mogelijk ook effecten heeft op de proliferatie van snel prolifererende eukaryote cellen, zoals de stellaatcel. Ribavirine is een competitieve remmer van inosine-5'-monophosphate dehydrogenase (IMPDH). IMPDH remmers reduceren de proliferatie van verschillende celtypen, inclusief tubulair epitheelcellen en mesangiale cellen in de nier, vasculaire gladde spier cellen, endotheel cellen, fibroblasten en myofibroblasten. In vivo zijn de IMPDH remmers anti-proliferatief en anti-fibrotisch in immuun-gemedieerde nierziekten.
We laten eerst zien dat ribavirine dosis-afhankelijk de proliferatie van geactiveerde stellaatcellen remt in vitro. Vervolgens hebben we een niet-viraal model van leverfibrose gebruikt in ratten, het galgang ligatie model (bile duct ligation: BDL). Dit model leidt tot fibrose binnen 10 dagen. Dagelijkse toediening van ribavirine reduceert verschillende parameters van fibrose en stellaatcelactivatie waaronder gereduceerde expressie van de stellaatcel activatie-merkers α-smooth muscle actin, TGF-β en het extracellulaire matrix eiwit collageen type I. Belangrijke vragen die nog beantwoord dienen te worden zijn de gevolgen van ribavirine behandeling op lange termijn en de effecten van ribavirine in het reduceren van al bestaande fibrose. Ook is het belangrijk om de gevonden veranderingen in genexpressie te verifiëren op eiwit niveau (matrix depositie en aantal geactiveerde stellaatcellen). Onze bevindingen kunnen een verklaring vormen voor het feit dat er bij sommige patiënten met een hepatic C virus infectie, behandelt met ribavirine, er wel een verbetering wordt gezien in de histologie, terwijl het virus niet compleet

verdwenen is. Onze bevindingen suggereren dat ribavirine mogelijk gunstige effecten heeft op de behandeling van niet-virale leverziekten.

Toekomst perspectief

In dit proefschrift hebben we de beschermingsmechanismen van geactiveerde stellaatcellen tegen oxidatieve stress-geïnduceerde toxiciteit belicht. We hebben belangrijke nieuwe informatie verkregen met betrekking tot de rol van anti-oxidanten, ROS-detoxificerende enzymen, glutathione en Mdr- en Mrp-transporters in de bescherming tegen oxidatieve stress. Verschillende van deze observaties dienen nader onderzocht te worden. Interessante nieuwe doelwitten voor interventie die voortkomen uit deze studies zijn Mrpl, gluthathione en ribavirine. Het is echter noodzakelijk dat interventies gericht moeten zijn op het juiste celtype. Vele interventies die de stellaatcel aanpakken zullen ook de hepatocyt of de progenitor cel treffen, hetgeen duidelijk niet gewenst is. In dit opzicht is de kennis van het specifiek richten van medicijnen op stellaatcellen erg belangrijk. Een "stellaatcel" gebaseerde interventie heeft ook het voordeel dat alle chronische leverziekten waarin fibrose zich ontwikkelt kunnen worden behandeld.

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Liefs, Sandra

Sandra Dunning werd geboren op 28 juli 1983 te Assen. Ze groeide op in het Drentse dorpje Hijken en later in Beilen. Haar havo-diploma behaalde zij in het jaar 2000 aan het Vincent van Gogh College te Assen.
Hetzelfde jaar begon zij aan haar HLO-opleiding aan de Hogeschool Drenthe te Emmen. Haar afstudeerstage heeft ze voltooid in 2004 bij de afdeling Klinische Genetica aan het Universitair Medisch Centrum te Groningen, toen der tijd nog genaamd het Academisch Ziekenhuis Groningen. Tijdens deze stageperiode is door middel van Comparive Genomic Hydridization Microarrray's (array CGH) gezocht naar een deletie hotspot in chromosoom drie in niertumoren, onder leiding van dr. Klaas Kok en dr. Eva van den Berg.
In 2004 is Sandra begonnen met haar promotie onderzoek bij de afdeling Maag-, Darm- en Leverziekten, onder leiding van prof. dr. Han Moshage en dr. Klaas Nico Faber. Het project getiteld 'Induction of stellate cell apoptosis: a novel therapy for liver fibrosis' werd mede gefinancierd door de Maag Lever Darm Stichting (registratienummer MWO 03-56). Het proefschrift dat voor u ligt, getiteld 'Resistance of activated stellate cells to cell death in liver fibrosis: mechanisms and targets for intervention' beschrijft de resultaten van dat onderzoek.

List of publications

Rebekka A. Hannivoort*, **Sandra Dunning***, Sara Vander Borght, Ben Schroyen, Jannes Woudenberg, Fiona Oakley, Manon Buist-Homan, Fiona A.J. van den Heuvel, Mariska Geuken, Albert Geerts, Tania Roskams, Klaas Nico Faber, Han Moshage. * These authors contributed equally to this work.
Multidrug resistance-associated proteins are crucial for the viability of activated rat hepatic stellate cells. *Hepatology 2008 Aug;48(2):624-34*

Sandra Dunning, Rebekka A. Hannivoort, Jan Freark de Boer, Manon Buist-Homan, Klaas Nico Faber,Han Moshage.
Superoxide anions and hydrogen peroxide inhibit proliferation of activated rat stellate cells and induce different modes of cell death. *Submitted*

Sandra Dunning, Rebekka A. Hannivoort, Jannes Woudenberg, Fiona A.J. van den Heuvel, Manon Buist-Homan, Klaas Nico Faber, Han Moshage.
Glutathione and antioxidant enzymes serve complementary roles in protecting activated hepatic stellate cells against hydrogen peroxide-induced cell death. *Submitted*

Rebekka A. Hannivoort, **Sandra Dunning**, Janny Wallinga, Kyrjon van Pelt, Manon Buist-Homan, Klaas Nico Faber, Han Moshage.
Ribavirin is anti-fibrotic in a non-viral rat model for liver fibrosis. *In preparation*